A New Qualitative RT-PCR Assay Detecting SARS-CoV-2

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Abstract

Purpuse: The world is facing an exceptional pandemic caused by SARS-CoV-2. To allow the diagnosis of COVID infection, several assays based on the real-time PCR technique have been proposed. The requests for diagnosis are such that it was immediately clear that the choice of the most suitable method for each microbiology laboratory had to be based on one hand, the availability of materials, on the other hand, the personnel and training priorities for this activity. Unfortunately, due to high demand, the shortage of commercial diagnostic kits has also become a major problem. To overcome these critical issues, we have developed a new qualitative RT-PCR probe based.

Methods: Our system detects three genes: RNA-dependent RNA polymerase (RdRp), envelope (E) and nucleocapsid (N), while the b-actin gene was used as an endogenous internal control.

Results: The results of our assay show complete agreement with those obtained using a commercially available kit, except for two samples that did not pass the endogenous internal control. The coincidence rate was 0.96. The LoD of our assay was 140 cp/reaction for N and 14 cp/reaction for RdRp and E.

Conclusion: Our kit was designed to be open either for the nucleic acid extraction step or on the RT-PCR assay to be carried out on several instruments. Therefore, it is free from the industrial production logics of closed systems and, conversely, it is hypothetically available for distribution in large quantities in any microbiological laboratory. The kit is currently distributed worldwide (called: MOLgen-COVID-19; Adaltis). A new version of the kit detecting S gene also is coming available.

Introduction

Since November 2019, the world has had to deal with an unprecedented public health emergency: the coronavirus: SARS-CoV-2, now COVID-19 [1, 2, 3]. The pandemic has put pressure on the entire health system and, above all, has put microbiologists in serious diagnostic difficulties, called upon to respond to medical needs without having valid scientific evidence, especially in the early stages [4–6]. Over time and through the gradual acquisition of new scientific findings, also supported by the World Health Organization (WHO) and the Centers for Disease Control (CDC) and the European Centers for Disease Control (ECDC), evidence about the virus has become increasingly available. Access to knowledge has made it possible to design diagnostic kits for the detection of viruses in biological samples. Many diagnostic systems have been proposed over the months that often differed in proposed genetic targets [7–11].

Gradually, the diagnostic systems passed the appropriate validations required by the Food and Drug Administration (FDA) or CE IVD. However, the massive global spread of the virus has caused an objective difficulty in the supply of diagnostic systems in the market, sometimes this is due only to viral nucleic acid extraction systems, others to amplification and detection systems, frequently both [9]. The shortage of diagnostic systems has imposed a consequent limitation on their use, this has presented serious difficulties in having test kits available, which later resulted in delays in the identification of positive

patients [12–14]. Unfortunately to date, even in our country, the problem still persists due to important differences between regions. On the one hand, we have to attend to the legitimate request of the population to access the testing and its availability; on the other hand, we have to face the same issue from the health institutions and make use of the technological and diagnostic resources available. Also, another key element is the problem of the shortage of human resources, engaged in microbiology laboratories to carry out COVID-19 tests.

After the moment of maximum spread of the virus, the real challenges for Italy and the rest of the world have turned to the storage of diagnostic tests, the simplicity in the administration of the latter to the population and the sustainability of the entire system. This aspect is particularly felt and relevant, especially now on the threshold of the second wave of diffusion [15].

At a time of great diagnostic difficulty, our research team designed a new diagnostic system that not only can meet the required sensitivity and specificity but also can perform a reasonably fast diagnosis so that it could be introduced into a diagnostic process at the height of the requests of the health system. Our assay (hereafter kit) is a probe-based qualitative reverse transcriptase real-time PCR (qRT-PCR) probe that detects COVID-19 target genes. In this paper, we present the characteristics of our system.

Materials And Methods

Specimens:

The leftover, remnants of specimens collected for routine clinical care (166 nasopharyngeal swabs), that would otherwise have been discarded, were used in the evaluation of our system. Samples were collected by trained personnel using nasopharyngeal Eswab™ (Copan, Brescia-Italy) and processed at Life GeneMap Laboratory Messina (Italy) using a commercially available system: Novel Coronavirus Real-Time Multiplex RT-PCR Kit (2019-nCoV) (3-gene detection) (Life River, San Diego, CA 92121, USA). The Life River system is one of those included in the list approved by the WHO (June 2020 WHO EUL Public Report, version 1.0) [16, 17].

Kit Design:

Our diagnostic assay is a probe-based qualitative reverse transcriptase real-time PCR (qRT-PCR) probe. The targeted COVID-19 genes detected by our assay are the RNA-dependent RNA polymerase (RdRp), the envelope (E), and the nucleocapsid (N) of COVID-19. The primers and probes were designed based on the published sequence of COVID-19 in NCBI (reference sequence NC 045512.2) and were synthesized by Bio-Fab Research (Bio-Fab Research, Rome, Italy). The two sets of primers are specific to COVID-19, respectively, the "E" primer for the Envelop gene and the "N" primer for the Nucleocapsid gene. One of them is called RdRp, which targets the polymerase gene and is common with the SARS virus. The concentrations of primers and probes have been determined by experimental procedures, and the sensitivity of the test carried out with the chimeric plasmid that is described below. Primer and probe sequences not shown as the kit is protected by intellectual property (MOLgen-COVID-19; SARS-CoV-2 Real Time RT-PCR kit, by Adaltis).

A portion of an endogenous human β-actin gene was used as an internal control (IC) for the test, the latter also allowing the evaluation of correct nasopharyngeal sampling.

To evaluate our kit, an initial proficiency-assay was carried out in the Microbiology laboratories of the Department of Experimental Medicine of the “Tor Vergata” University of Rome. This proficiency-assay was run using chimeric plasmids (CPs) in which virus sequences were artificially inserted into a plasmid [pBlueScript II SK(+)]. Synthesis of CP was contracted for Bio-Fab Research (Bio-Fab Research, Rome, Italy).

The analytical specificity and cross-reactivity of the primers and probes in our kit were evaluated using ZeptoMetrix panels (ZeptoMetrix, Co., Buffalo, NY, USA): a) NATtrolTM SARS-CoV-2 (E/ORF/1ab recombinant) Stock (ZeptoMetric); b) NATtrolTM SARS-CoV-2 (recombinant only N) stock; c) NATtrol TM Coronavirus-SARS Stock. Stock, which is formulated with intact and purified bacterial cells that contain synthetic SARS-CoV-2 sequences (the cells have been chemically modified to render them non-infectious and stable in the refrigerator) (Table 4). The panels are supplied in a purified protein matrix that mimics the composition of real clinical samples. Cross-reactivity was evaluated using: a) NATtrol ™ Respiratory Verification Panel (Zeptometrix NATRVP2-BIO) containing 22 viral and bacterial targets; 2) NATtrolTM MERS-CoV Stock (NATMERS-ST), both panels contain intact viral and/or bacterial particles chemically modified to render them non-infectious and stable in the refrigerator.

The analytical sensitivity (LOD) of the test was determined by serial dilution with the AccuPlexTM SARS-CoV-2 v2 Reference Material Kit containing 5175 RNA cp/mL of inactivated SARS-CoV-2 virus, and the Research reagent for SARS-CoV-2 RNA (NIBSC code 19/304) obtained from the National Institute for Biological Standard and Control (NIBSC, UK). We treated the samples from both panels as a common nasopharyngeal sample, but in triplicate (Table 5).

Assay conditions:

A 200µl aliquot of samples collected in EswabTM was extracted, both with a manual procedure using the magnetic silica bead procedure (MOLgen Universal Extraction Kit, QIAamp viral RNA) according to the manufacturer's instructions. To process many samples at once, the extraction procedure was also automated at ExtraLab (Adalties Srl, Guidonia, Italy).

Real-time amplification was performed in double on the AmpliLab system (Adalties Srl, Guidonia, Italy) and CFX96 (Bio-Rad, Hercules, CA, USA) using qPCRBIO PROBE 1-Step Go No-Rox (PCR bio-systems; www.pcrbio.com). To establish the appropriate amount of reverse transcriptase activity, RTase Go quantitation was performed according to the manufacturer's instructions. The titration experiment showed that 0.2µl of 20X RTase Go, in the amplification mix, gave good results in terms of sensitivity.

We achieved the ideal reaction condition using the 20µl reaction a master mix composed as follows: 2X qPCRBIO probe 1 step Go Mix 10µl; ppMix (a mixture of all primer and probe) 5µl; 20X RTase Go 0.2µl; and 5µl of a sample. Particularly, ppMix contained as a final concentration: 10 picomoles of RdRp, E and

β -actin (forward and reverse primers), 30 picomoles of N (forward and reverse primer); 2.5 picomoles of the probe for each target.

The RT-PCR conditions, for both instruments, were: one step at 45°C for 10 minutes; a step at 95°C for 2 min; 40 steps at 95°C for 5 seconds and the last one at 60°C for 25 seconds.

The instrument was programmed to read the RdRp gene in Fam, the E gene in Rox, the N gene in Cy5, and β -actin in the Hex channel.

The kit is now worldwide distributed by Adaltis and the commercial name is MOLgen-COVID-19 Real Time RT PCR.

Upcoming updates: The kit has been recently implemented with and additional gene: S (spike gene) which is detected on Cy5-5 channel. The evaluation on clinical sample is still ongoing (data not shown). Figure 4 reports the curve and relative CT of a positive sample.

All methods described were carried out in accordance with relevant guidelines and regulation and the study was approved by Independent Ethics Committee Tor Vergata Polyclinic "on 25 June 2020.

Results

Positivity in our test was based on WHO guidelines [7]. Particularly, a sample was considered positive if it showed a signal in at least one Rox (E gene) and/or Cy5 (N gene) fluorophore, while the presence of a single positive signal in the Fam channel was considered "inconclusive", the RdRp gene being designed to be common with other Sarbecovirus. Contrarily, the absence of signal in all channels, with the exclusion of Hex (that of β-actin), allowed us to conclude a sample as negative. The absence of a signal in all channels allowed us to conclude a sample as "invalid" due to probable inhibition of the PCR reaction or unreliable sampling. Table 1 reports the interpretation criteria. Figure 1 reports the curve and relative CT of a positive sample.

The results of our test showed the absence of any cross-creation, and evidence of a specific reaction of our primers and probes towards the COVID-19 genes: the data are shown in Table 3, 4, and Fig. 2. The limit of detection (LoD) of our kit is shown in Table 5. The mean CT value for amplification of the β-actin gene in a negative sample was 27,81 ± 3.06.

Diagnostic sensitivity and coincidence rate were evaluated by testing our kit with the Coronavirus SARS-CoV-NAT positive panel (catalog nr NPP-COV-001) supplied by Biomex (Biomex, GmbH Germany). The panel comprises 20 individual donor members containing coronavirus RNA inactivated in viral transport medium (VTM). Each member was tested in triplicate after RNA extraction using our kit, the Coincidence rate was 0.96.

The study included 166 samples. 133 samples were negative, 31 were positive, two samples did not show amplification of the endogenous internal control (β-actin) and we concluded it as indeterminate. Figure 3

shows examples of positive and inconclusive samples.

Of the 31 positive samples, 6 were only positive for RdRp and therefore reported as "inconclusive" and 25 were positive for COVID-19. Among these 25, nine were positive for all objectives, while 16 RdRp and E only (see Table 2). The "inconclusive" samples were retested, and the results confirmed the detection of a single target (RdRp) that is designed to be common with Sarbecovirus. The CTs recorded in the Fam channel (RdRp) for these six samples were: 33.86; 29.36; 36.52; 29.87; 36.51; 35.28

The results shown by our assay agreed with those obtained by using the Novel Coronavirus (Novel-Cov19) Multiplex Real-Time RT- PCR (RR-0479-02) (LifeRiver Bio-Tech; US) for the initial diagnosis of SARS-CoV-2.

Discussion

Accurate and reliable diagnostic analysis and large-scale testing are essential for the early detection of pathogens related to disease outbreaks, but it is even more important to take timely public health actions during pandemic events. This has proven to be true for SARS-CoV-2, which was identified as the cause of an outbreak of pneumonia in Wuhan, China in December 2019, and rapidly spread around the world [18–25]. Laboratory diagnosis of infections caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is achieved primarily by performing nucleic acid amplification tests (NAAT) on samples from the respiratory tract. Indeed, upper respiratory tract specimens, such as nasopharyngeal swabs and oropharyngeal swabs, generally have elevated SARS-CoV-2 viral loads at the onset of symptoms [25]. Some authors have recently suggested expanding NAAT testing to include saliva and stool samples, but the debate is still ongoing [26, 27]. Due to the urgent worldwide request for tests to diagnose COVID-19, many NAAT assays for SARS-CoV-2 are available and many others are in the final stages of development [24, 28, 29]. This aspect has the great advantage of making a wide range of diagnostic tests available to health systems and therefore allows us to respond to the diagnostic needs caused by the pandemic. On the other hand, the staggering number of kits available around the world coupled with the differences between the NAAT assays pose problems in the validation process itself. An important limit in qRT-PCR validation assays, which detect SARS-CoV-2, is the availability of standard RNA-viruses. Also, it is the subject of an open discussion whether or not to consider the full-length integral SARS-CoV-2 RNA as a safety level 3 biohazard [23, 24]. If the debate reaches a consensus to mark SARS-CoV-2 as Level 3, its treatments could only be performed by laboratories with adequate level 3 (BSL3) security measures, but this limits the ability to perform experimental tests [28]. With these critical issues in mind, we have developed a qRT-PCR assay capable of detecting three SARS-CoV-2 target genes. The strongest aspect of our assay resides in the IC, being a β-actin a conserved gene present in all human cells, its detection can be useful to establish the reliability of the sampling. In fact, the accurate collection of nasopharyngeal samples has revealed a crucial aspect of the pre-analytical phase that strongly conditions the results of the NAAT test, being one of the most frequent and probable causes of false-negative results and therefore of a late diagnosis [18, 28, 30]. Also, to avoid working with a full-length viral RNA, overcoming the problem of BSL3, we have chosen to build an artificial chimeric plasmid to test our primers and probes,

while using ZeptoMetrix panels that allowed us to evaluate the specificity of our assay under safe conditions.

Another powerful aspect of our kit is that it is intended to be open, either for the nucleic acid extraction step or in the RT-PCR assay that will be performed on several instruments (in this paper we tested two of them). Therefore, our assay can be used in any molecular biology laboratory. Also, our kit is free from the industrial production logic of "closed systems" and, conversely, is hypothetically available for distribution in large quantities. This aspect, at a time of great demand for tests and equally well-known shortcomings of commercial kits, can be a significant strength in facilitating introduction into microbiological laboratories [31].

Moreover, considering the potential genetic drift of SARS-CoV-2, especially as the virus evolves within new populations, although the literature suggests that at least two specific molecular targets should be included in the assay to reduce the probability for cross-reactions, we have implemented our kit by adding a forth target gene codifying for glycoprotein spike (S) S gene, but validation tests are still in progress [28, 29, 32, 33].

Finally, as evidenced in the literature shows, besides direct respiratory sampling, rectal swabs and saliva may be suitable specimens to enhance the diagnosis of COVID-19, and we are expanding our assay validation test in this direction [27, 28].

Declarations

Funding

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Conflicts of interest/Competing Interests

Carla Fontana has received a research grant by Quintiles/Angelini. Advisory Board: Angelini, Pfizer

Marco Favaro has received a research grant by Alifax R&D and Adaltis s.r.l

Walter Mattina, Simon Middleton, Silvia D'Angelo and Tullio Altarozzi are employed in Adaltis

The remaining authors have no competing interests.

Ethics approval

The study did not include human participants but leftover samples. Specific informed consent are not required (as stated by "Independent Ethics Committee Tor Vergata Polyclinic "on 17 June 2020), having based this study on the use of leftover human specimens collected for routine analysis that would otherwise been discarded. The same specimens are "unlinked anonymized materials". This statement is

in agreement to FDA “Guidance on Informed Consent for In Vitro Diagnostic Device Studies Using Leftover Human Specimens that are Not Individually Identifiable” April 25, 2006, and “Bioethical ed use die campion biologic umani” Pezzoli P. & Graziani MS biochemical clinic, 2008, vol. 32, n. 3.

Availability of data and material

All data are provided in full in the results section of this paper. This paper was preprint from MedRxiv **https://www.medrxiv.org/content/10.1101/2020.06.17.20124396v1**

Authors’ contributions

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Tables

Table 1
Interpretation criteria used in our qRT-PCR assay

Target gene and channel	Example 1	Example 2	Example 3	Example 4	Example 5	Example 7
IC (b-actin) HEX	+/-	+/-	+/-	+/-	+	-
RdRp gene (FAM)	+	+	+	+	-	-
E gene (ROX)	-	+	+	-	-	-
N gene (Cy 5)	-	+	-	+	-	-
S gene (Cy 5.5)[a]	-	+	+/-	+/-	-	-
Results interpretation	Negative for CoVid19 Sarbecovirus detected	Positive for CoVid 19	Positive for CoVid 19	Positive for CoVid 19	Negative	Sample invalid probably inhibition or unsuitable withdrawal
Note: Cut off value for all gene is ≤ 40; In presence of strong signal of others genes, the signal of IC may be inhibited but the results still valid						
In agreement with WHO guideline a sample is defined positive in presence of at least one specific gene among those detected (N, E, S); in our kit RdRp is in common with other Sarbecovirus						
a = S gene has been recently added to our kit						

Table 2
Results obtained by our assay (qRT-PCR)

Results	**Target gene**				**No. sample**
	RdRp	N	E	β-actin	
Indeterminate	-	-	-	-	2
Inconclusive	6			6	6
Positive	9	9	9	9	9
	16		16	16	16
Negative				133	133
Total					166
S gene was not present in our kit at the time of the initial evaluation here reported					

Table 3
Cross reactivity evaluation using NATtrol™ Respiratory Verification Panel (Catalog Number NATRV2-BIO) and NATtrolTM MERS-CoV Stock (Catalog Number NATMERS-ST)

Organism	Strain	Results (RdRp + E + N)
Influenza A	H1N1 A/New Caledonia/20/99	Undetected
Influenza A	H3 A/Brisbane/10/07	Undetected
Influenza A	2009 H1N1 A/NY/02/09	Undetected
Influenza B	B/Florida/02/06	Undetected
Metapneumovirus8**	Peru6-2003	Undetected
Respiratory Syncytial Virus A	N/A	Undetected
Rhinovirus 1A	N/A	Undetected
Parainfluenza virus Type 1	N/A	Undetected
Parainfluenza virus Type 2	N/A	Undetected
Parainfluenza virus Type 3	N/A	Undetected
Parainfluenza virus Type 4	N/A	Undetected
Adenovirus Type 3	N/A	Undetected
M.pneumoniae	M129	Undetected
C. pneumonia	CWL-029	Undetected
C. pertussis	A639	Undetected
Adenovirus Type 31	N/A	Undetected
Adenovirus Type 1	N/A	Undetected
B. parapertussis	A747	Undetected
Coranovirus NL63	N/A	Undetected
Coronavirus 229E	N/A	Undetected
Coranovirus OC43	N/A	Undetected
Coronavirus HKU-1	N/A	Undetected
MERS-CoV	Florida/USA-2Saudi Arabia_2014	Undetected

Table 4
Specifity evaluation of our kit using NATtrol™ SARS-CoV-2 (E/ORF1ab recombinant), NATtrol™ SARS-CoV-2 and NATtrol™ Coronavirus SARS

Organism	Results target RdRp	Results target E	Results target N
SARS-CoV-2 (recombinant)	Undetected	**Detected**	Undetected
SARS-CoV-2 (recombinant only N)	Undetected	Undetected	**Detected**
Coronavirus SARS	**Detected**	Undetected	Undetected

Table 5
Limit of Detection (LoD) of our assay evaluated using Research Reagent for SARS-CoV-2 RNA (NIBSC code 19/304) from the National Institute for Biological Standard and Control

NIBSC	Dilution Factor	Cp/mL	Cp/Reaction	target RdRp	target E	target N
NIBSC code 19/304	1 to 1000	10000	140	31.58	30.4	38.3
NIBSC code 19/305	1 to 2500	4000	56	32.9	31.4	-
NIBSC code 19/306	1 to 10000	1000	14	34.78	32.63	-

Figures

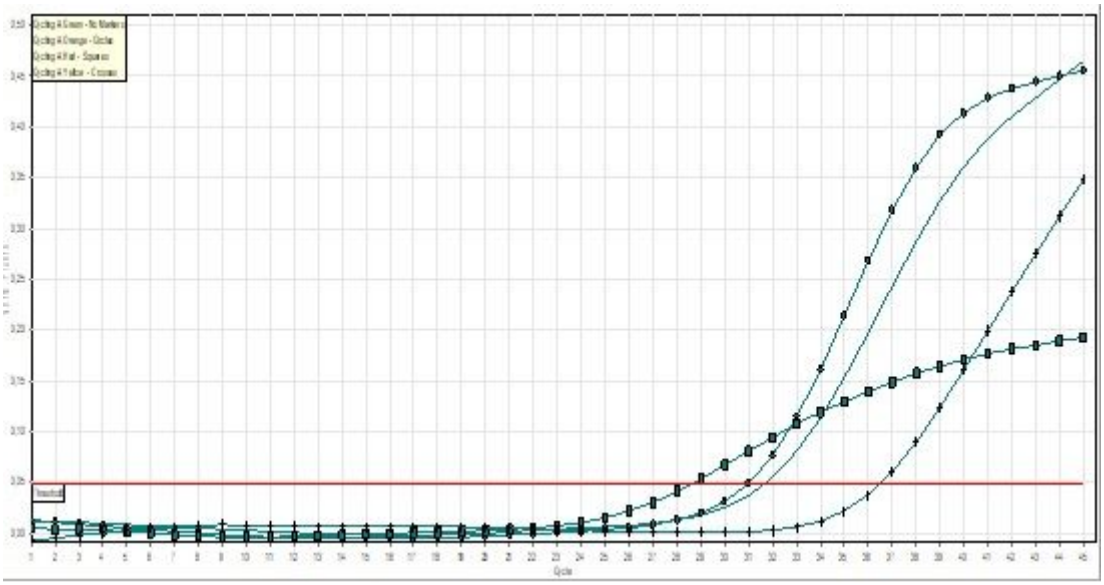

Figure 1

The figure illustrates the Real Time PCR curves of the genes detected by our kit; RdRp gene: RNA-dependent RNA polymerase gene; E: the envelope gene; N: the nucleocapsid gene of COVID-19. IC represents Internal Control (b-actin gene)

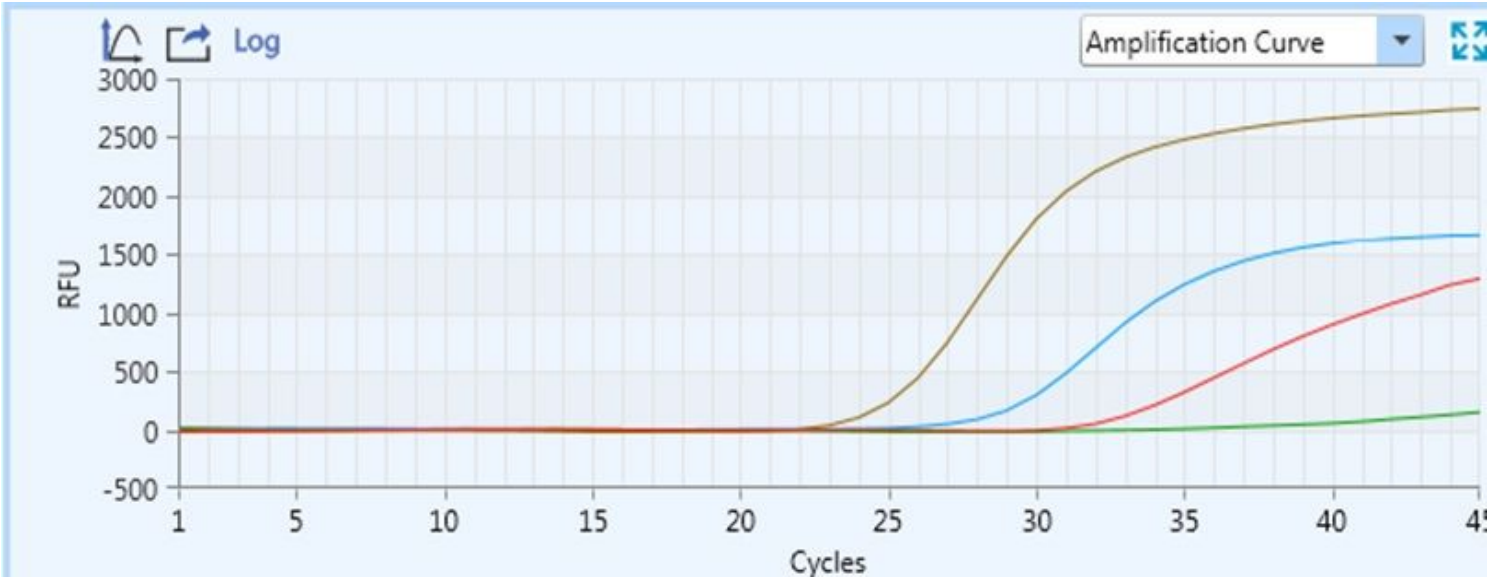

Figure 2

The figure illustrates the Results of qRT-PCR assays obtained using ZeptoMetrix TM panels. On y axis: Relative Fluorescence Unit (RFU); on x axis: threshold cycle (CT)

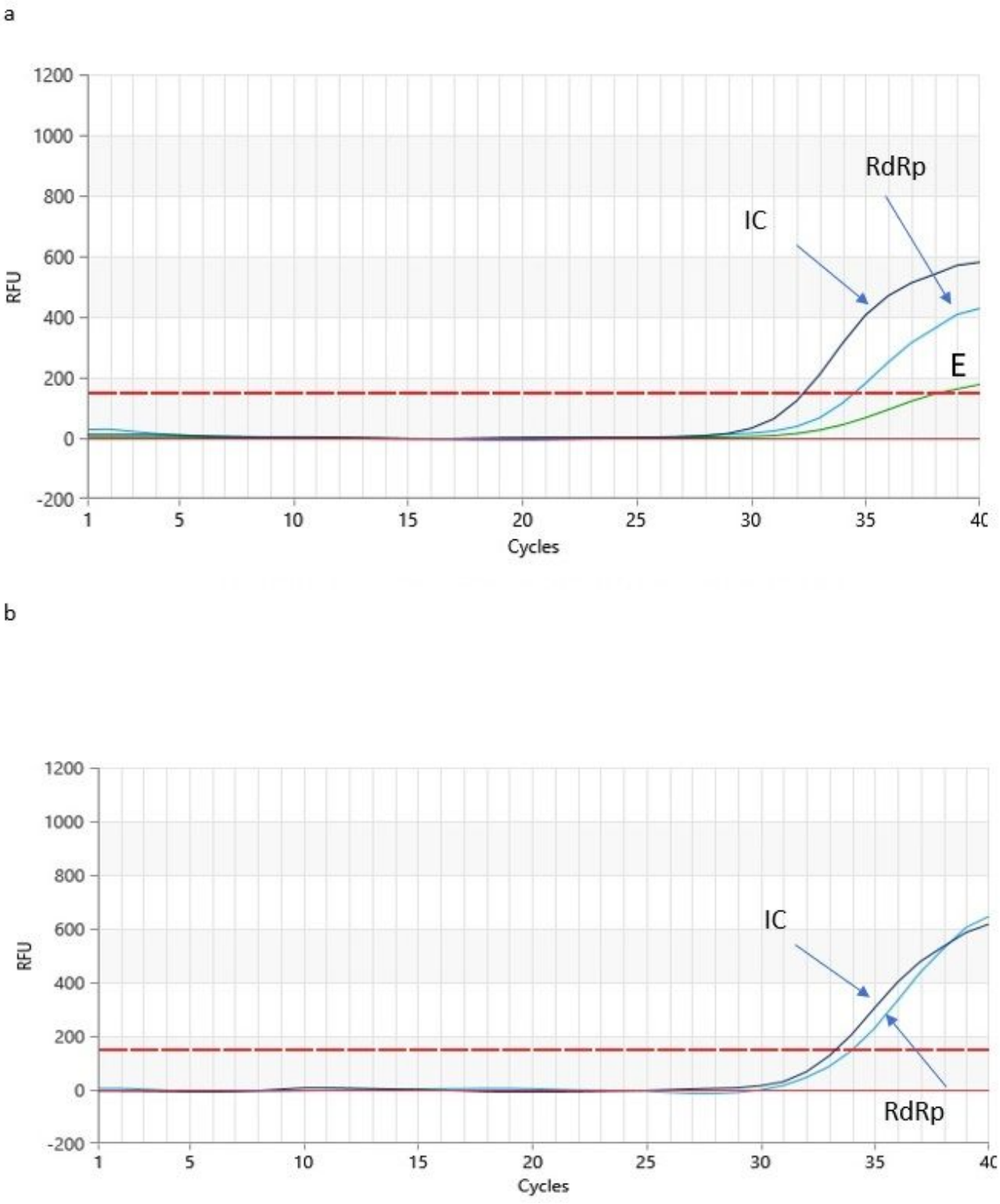

Figure 3

Box A shows a positive sample in which has been detected two target genes (RdRp and E) Box B shows a sample concluded as inconclusive being detected RdRp only

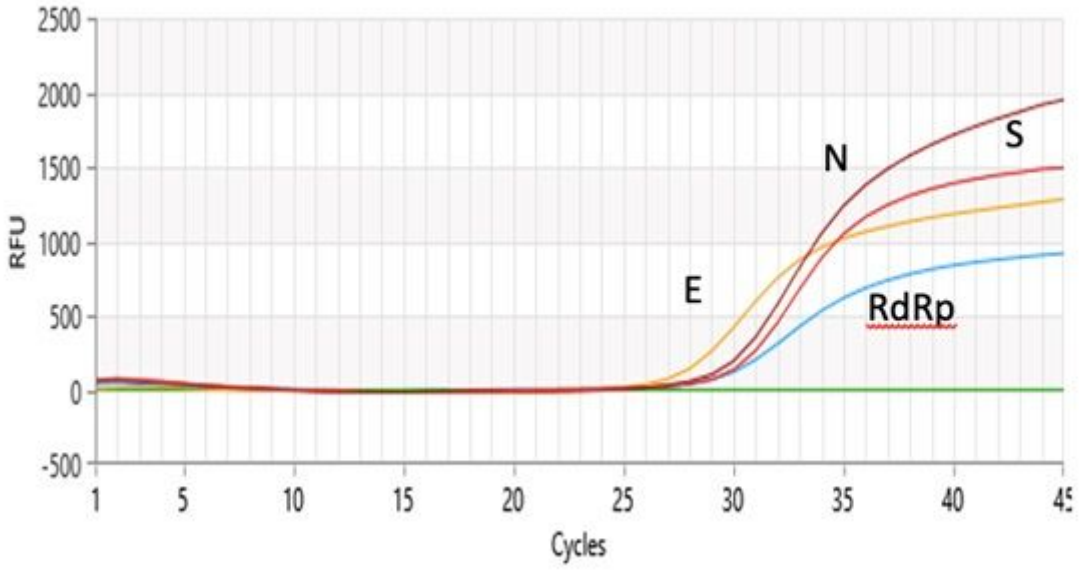

Figure 4

The figure illustrates the Real Time PCR curves of the genes detected by our kit; RdRp gene: RNA-dependent RNA polymerase gene; E: the envelope gene; N: the nucleocapsid gene; S: spike gene of COVID-19