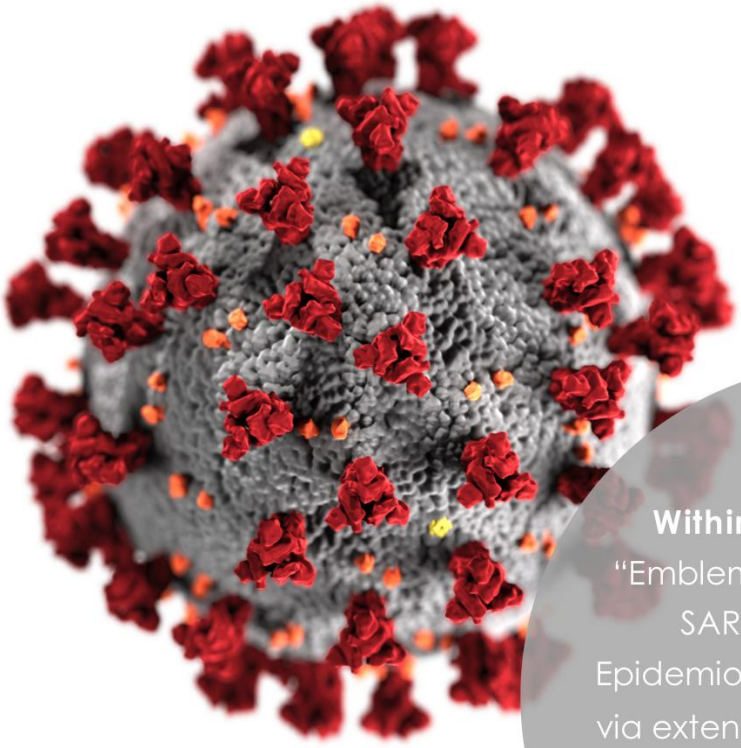
HANDBOOK OF "IN HOUSE" MOLECULAR AND IMMUNOLOGICAL METHODS TO DETECT SARS-CoV-2



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Within the frame of the:
"Emblematic action to handle SARS-CoV-2 infection: Epidemiological study in Greece via extensive testing for viral and antibody detection, sequencing of the virome and genetic analysis of the carriers"

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"Emblematic action to handle SARS-CoV-2 infection: Epidemiological study in Greece via extensive testing for viral and antibody detection, sequencing of the virome and genetic analysis of the carriers"

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1. Introduction

1.1 Sars-CoV-2 Pandemic

Coronaviruses, belong to the family of *Coronaviridae* and can cause respiratory and neurological diseases. Until 2019, six human coronaviruses (HCoVs) had been identified **[Cheng *et al.*, 2020]**. In particular these HCoVs are widely known as HCoV-229E, HCoV-HKU1, HCoV-OC43, HCoVNL63, severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) **[Liu *et al.*, 2020].** For reasons yet to be explained, HCoVs can cross species barriers and can cause, in humans, illness ranging from a common cold to more severe diseases. At the end of December 2019 many cases of a novel type of pneumonia was reported in Wuhan, Hubei Province in China. This strange phenomenon rapidly evolved as a global pandemic which was named Coronavirus disease 2019, COVID-19 **[Wang *et al.*, 2020]**. Scientists around the world identified that the disease was caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). As of July 28, 2020 more than 16.672.136 cases, 657.262 deaths and 10.262.387 cases of recovered patients have been reported.

The emerging global pandemic condition posed a serious problem on human public health. Thus, it is vital for the scientific community to take action for a putative future recurrence of this situation. Such conditions require effective, among others, molecular identification and detailed characterization of the virus, extensive description of clinical features, in depth understanding of the pathophysiology of the disease and precise definition of viral targets in order to achieve the development of vaccines as well as efficient antiviral drugs.

Based on the international experience and the guidelines from the World Health Organization (WHO) the detailed understanding of epidemic conditions requires a thorough investigation of the biological traits of the causing virus and particularly accurate characterization of its genome **[Yi *et al.*, 2020]**. Viruses have the ability to multiply in large numbers within human cells and therefore accumulate mutations at fast rates. Nonetheless, most of them appear to result in the inactivation of the virus or may be neutral, with no effect on the virus. Yet, few of them increase the infectivity or cytotoxicity of the virus with detrimental effects not only for the host in which they develop but also in individuals that are secondary infected by the initial host.

1.2 Counteracting actions

It is now well established that the evolving pandemic of SARS-CoV-2 represents a major, public health issue, worldwide. Towards this, it is imperative in the face of a pandemic condition and considering the possibility of re-appearance of the virus to deploy and to make available the appropriate molecular/immunological diagnostic tools for population screening, detection of the infected people and finally control of inter-personal contacts with the affected individuals.

Within this frame worldwide, often collaborative, attempts have been developed to design appropriate molecular and immunological tools that will facilitate such tasks. The current handbook describes the actions undertaken in Greece to develop such “in house” methods within the frame of the *Emblematic action to handle SARS-CoV-2 infection: Epidemiological study in Greece via extensive testing for viral and*

antibody detection, sequencing of the virome and genetic analysis of the carriers." under governmental auspices.

1.3 The: "Emblematic action to handle SARS-CoV-2 infection: Epidemiological study in Greece via extensive testing for viral and antibody detection, sequencing of the virome and genetic analysis of the carriers"

Similar to other actions undertaken in different countries an emblematic action is implemented in Greece in order to provide the means and to help cover needs for extensive viral and antibody detection, sequencing of the virome and genetic analysis of the carriers. This action is a multi-center Greek coalition, with multilayered and interconnected activities that received governmental support. The development of "in house" methods in order to cover potential increasing demands for molecular and immunological testing is among the main scopes of this action. The molecular testing focuses on the identification of the viral RNA, while the immunological includes the detection of antibodies against the virus in patients' blood serum as well as the development of monoclonal antibodies to identify the virus in biological samples. Another important perspective is to perform epidemiological studies resulting in the detailed mapping of mutations of the viral genome within the Greek territory, in parallel with the genomic analysis of the host. These approaches are anticipated to shed light on genetic features, putatively associated with the high prevalence of infection, clinical severity and response to therapies. These actions will allow time-related tracking and geographical mapping of the virus, allowing the State to make decisions and to proceed with appropriate public health interventions that will prevent the spread of the virus and ensure public health.

2. SARS-CoV-2 infection and immunological response

2.1 Epidemiology, viral structure: genome/proteins, life cycle into humans

SAR-CoV-2 is an enveloped, non-segmented, positive-sense single-stranded RNA (ssRNA) virus. According to phylogenetic analysis, its genome exhibits high homology (up to 80%) with SARS-CoV and MERS-CoV [**Weiss *et al.*, 2011; Corman *et al.*, 2018**] with a size between 26.4 to 31.7kb and a diameter of about 65–125nm. It is also composed of two untranslated regions (UTRs) at the 5' and 3' ends and a variable number (6-11) of open reading frames (ORFs) which encode 27 different proteins responsible for the replication and infectivity of the virus **(Figure 1) [Helmy *et al.*, 2020; Guo *et al.*, 2020].**

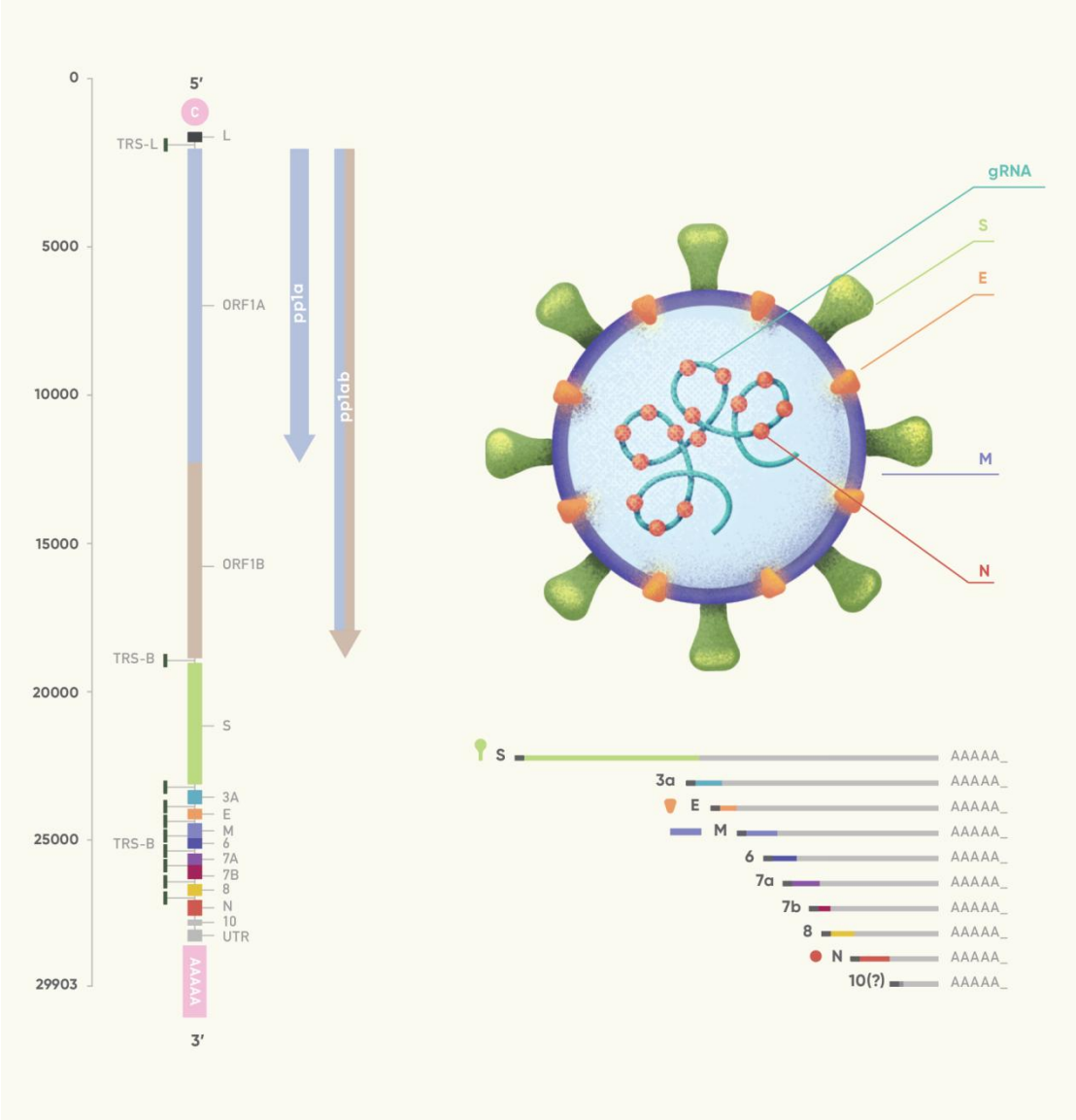


Figure 1. *Schematic Presentation of the SARS-CoV-2 Genome Organization, the Canonical Subgenomic mRNAs, and the Virion Structure [**Kim et al., 2020**]*

During cellular infection, the genomic RNA is translated from two open reading frames (ORFs), ORF1a and ORF1b, which encode nonstructural proteins (nsps) **(Figures 1, 2)**. The subgenomic regions comprising these ORFs are highly conserved

between Coronavirinae subfamily members and consist of pp1a and pp1b genes encoding the Polyprotein 1a and ab, respectively. Both Polyprotein 1a and ab are cleaved producing 11 and 16 nsps, respectively by a reaction which is catalyzed by viral proteases nsp3 and nsp5 [**Kim *et al.*, 2020; Helmy *et al.*, 2020**]. Nsp12 is a key molecule which acts as an RNA-dependent RNA polymerase (RdRp) indicating that the viral RNA is used as template for virus replication. Downstream of this conserved region the remaining subgenomic region encodes genes for structural viral proteins. Using cell's own machinery, SARS-CoV-2 synthesizes its positive-sense genomic and subgenomic RNAs, the latter encoding conserved structural proteins (spike protein [S], envelope protein [E], membrane protein [M], and nucleocapsid protein [N]), and 6 additional accessory proteins [**Kim *et al.*, 2020**].

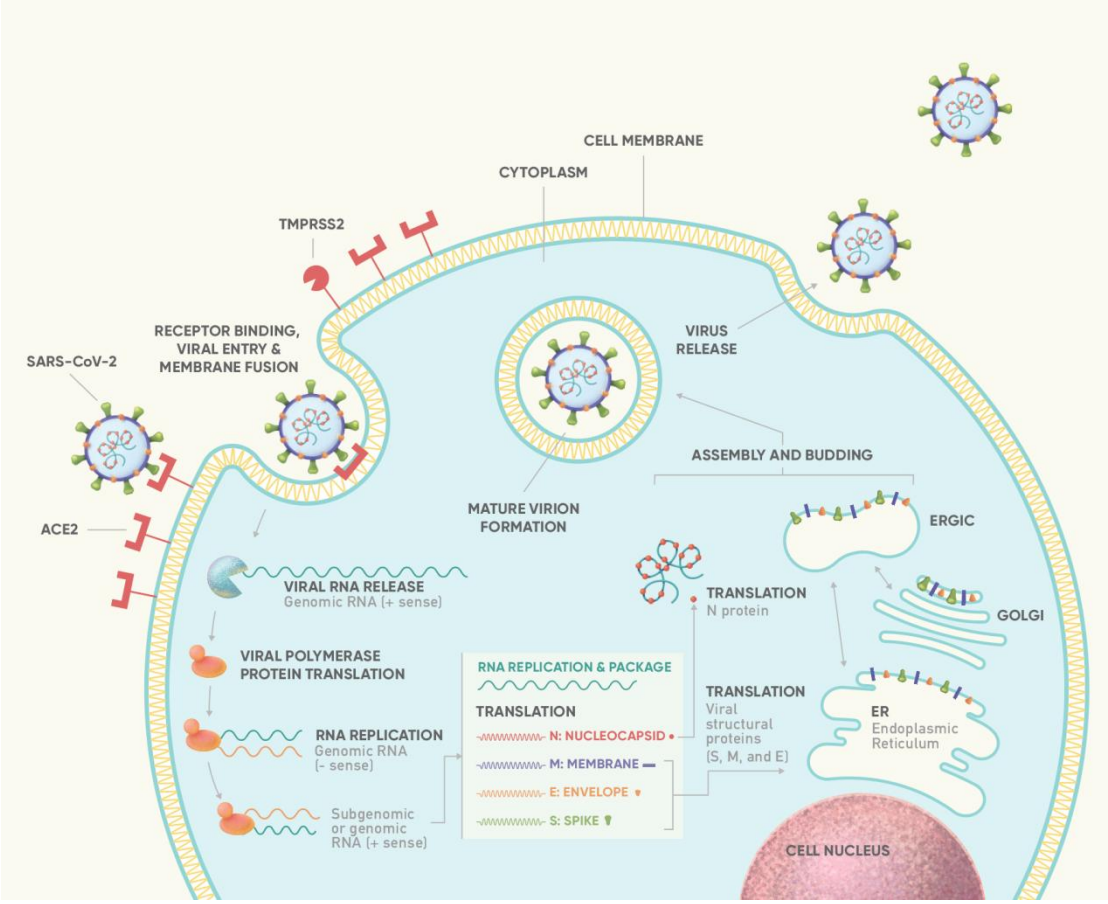


Figure 2. *Life cycle of highly pathogenic human coronaviruses (CoVs) [**Jiang et al., 2020**]. Binding of S protein to ACE2 receptor induces the proteolytic cleavage of S protein by cell surface-associated transmembrane protease serine 2 (TMPRSS2) and cathepsin. S1-RBD is recognized by ACE2 [**Shereen et al., 2020**] changing the structure of S protein, mediating virus fusion and entry into the target cell via endocytosis [**Jiang et al., 2020; Alanagreh et al., 2020**]. Following entry into host cell, the virus releases its positive-sense RNA-strand into the cell cytoplasm. Using ribosomes of infected cell, virus ORF1a and ORF1ab are translated producing the pp1a and pp1ab polyproteins. Positive (+)-sense RNA genomes serve as templates to produce full length of negative (-)-sense RNAs of virus genome by a reaction catalyzed by RTC. Replication of viral RNA and N protein is then followed. N protein is transcribed and produced into the cytoplasm mediating the packaging of viral RNA. At the same time, genes which encode S, M and E protein are transcribed and translated in the endoplasmic reticulum and transported to Golgi complex to form ER–Golgi intermediate*

*compartment (ERGIC). Virus assembly and budding is mediated through the interaction between the RNA-N complex and the ERGIC creating a mature virion. Finally, the newly formed virion is released from host cell via exocytosis [**Jiang et al., 2020; Tu et al., 2020; Alanagreh et al., 2020**].*

Spike protein (S) consist of two subunits, the N-terminal half (S1) and C-terminal half (S2). S1 domain also contains the Receptor Binding domain (RBD) of the virus. Each domain is responsible for a different process. Angiotensin-converting enzyme 2 (ACE2) is used as a receptor by the virus and RBD is the key domain of S protein responsible for binding of virions to the receptor of host cells [**Du *et al.*, 2020**].

S2 subunit has a key role in the process of fusion between the virus and the cell membrane acting as a class I viral fusion protein [**Walls *et al.*, 2020**]. Also, stabilization of fusion machinery complex is supported by the RBD, making it a crucial molecule for the infection and a potential therapeutic target.

The envelope protein is an oligomeric protein located at the membrane of SARS-CoV-2 acting as viroporin creating a pentameric protein-lipid ion transport channel [**Schoeman *et al.*, 2019; Ruch 2012**].

Membrane protein (M) is a glycoprotein and the most abundantly expressed one in infected host cells. It mediates the viral assembly and maturation via protein interaction with the structural viral proteins shaping the virions [**Tseng *et al.*, 2020**].

Nucleocapsid (N) protein is a dimeric protein which interacts directly with the RNA of the virus and provides the genomic stability necessary for the RNA transcription and replication [**Yoshimoto 2020**]. The main role of N protein is the packaging of RNA into a nucleocapsid structure or ribonucleoprotein (RNP) complex [**Kang *et al.*, 2020**] promoting the viral release [**Zeng *et al.*, 2020**].

2.2 Immunological response against SARS-CoV-2

Following receptor binding and internalisation into the host cell, a cascade of events takes place that activate and mount an immune response against SARS-CoV-2 that includes both the innate and adaptive arms of our immune system (**Figures 3, 4**).

Infected cells release newly formed viral particles and associated molecules (e.g ssRNA) along with host cell's substances such as ATP that act as danger signals activating the immune system. Both the former called PAMPs (Pathogen Associated Molecular Patterns) and the latter called DAMPs (Damage Associated Molecular Patterns) activate tissue resident innate immune cells (mainly dendritic cells, alveolar macrophages) via receptor-mediated recognition. More specifically, Toll Like Receptors (TLRs) recognise the above signals outside the cell, while cytosolic Retinoic acid-Inducible Gene-I (RIG-I)-Like Receptors (RLRs) and Nucleotide-binding Oligomerization Domain (NOD)-Like Receptors (NLRs) identify them intracellularly [**Kawai *et al.*, 2009**; **Li *et al.*, 2020; Perry *et al.*, 2005**].

Infected cells mount a type I Interferon response, releasing IFNα/β which primes nearby unaffected cells as well as immune cells to mount an anti-viral immune response through IFNγ release [**Fitzgerald-Bocarsly *et al.*, 2007; Ivashkiv *et al.*, 2014; Darwich *et al.*, 2009**]. Tissue's most potent type I IFN responder are dendritic cells which augment alveolar macrophage and NK-cells activity [**Ali *et al.*, 2019**]. This leads to IFNγ release which exerts its antiviral properties locally by supressing

viral replication and dissemination (systemically), acting in concert with other IFN family members (**Figure 3**) [**Schroder *et al.*, 2004**].

Once the above cells (alveolar macrophages, NK, dendritic cells) are activated, they release cytokines (such as TNF-a, IL1β, IL6, IL8, chemokines) that amplify and propagate the immune response by chemotactically recruiting more cells in the following time sequence. Neutrophils are recruited first (within minutes to 12-24 hours post-infection, usually peak at day 2-3) followed by monocytes, T-cells and B-cells that appear from day 4-5 onwards, thus switching to the adaptive arm of the immune response.

Monocytes differentiate into macrophages once in the affected tissue and along with tissue-resident phagocytic cells (dendritic cells and macrophages) phagocytose and antigen-present SARS-CoV-2 N and S proteins to cells of the adaptive immunity, namely T-cells (CD4+ T-helper, both Th1 and Th2 [**Ye *et al.*, 2020**] and CD8+ T-cytotoxic) and B-cells.

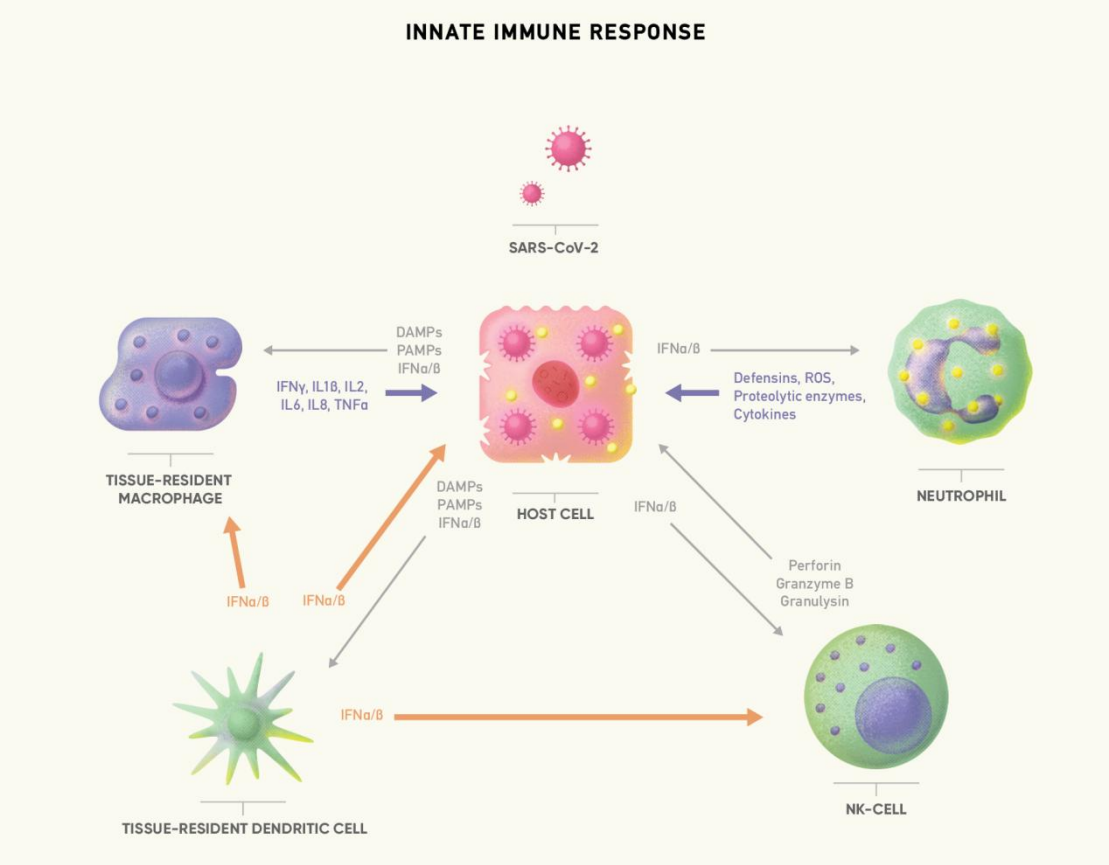


Figure 3. *Upon viral entry into host cells, the innate arm of the immune response is activated to eliminate the virus and decrease tissue damage. Tissue damage signals along with Type I IFN (interferon) responses of infected and nearby cells activate, prime and augment actions of dendritic, NK-cells and macrophages. Effector molecules including cytokines and chemokines are shown. Thick arrows represent pronounced immune cell involvement/contribution in SARS-CoV-2 innate responses. Of note, in severe/critical COVID-19 cases, NK-cell's killing action appears reduced secondary to immune exhaustion [**Yaqinuddin et al., 2020**]. For more detailes, see relative text.*

It has been found that effector CD8+ cytotoxic T-cell responses are more potent than those mediated by CD4+ T-cells in infections from coronaviruses [**Li *et al.*, 2020**]. On one hand, CD8+ cytotoxic T-cells recognise infected cells through MHCI interactions which makes them exert their cytotoxic actions via Perforin, Granzyme B and granulysin release. On the other hand, CD4+ T-helper cells – especially of the Th1 subpopulation - assist the effects of CD8+ T-cells while their Th2 subpopulation assist in B-cell activation and differentiation into plasma cells; the latter being responsible for the humoral immune response [**Romagnani *et al.*, 2000**].

Although variable (**Table 1**), antibodies against N and S viral proteins start to increase approximately 10 days after symptom onset, while seroconversion of most patients is being achieved within 3 weeks **(Figure 4)** [**To *et al.*, 2020**].

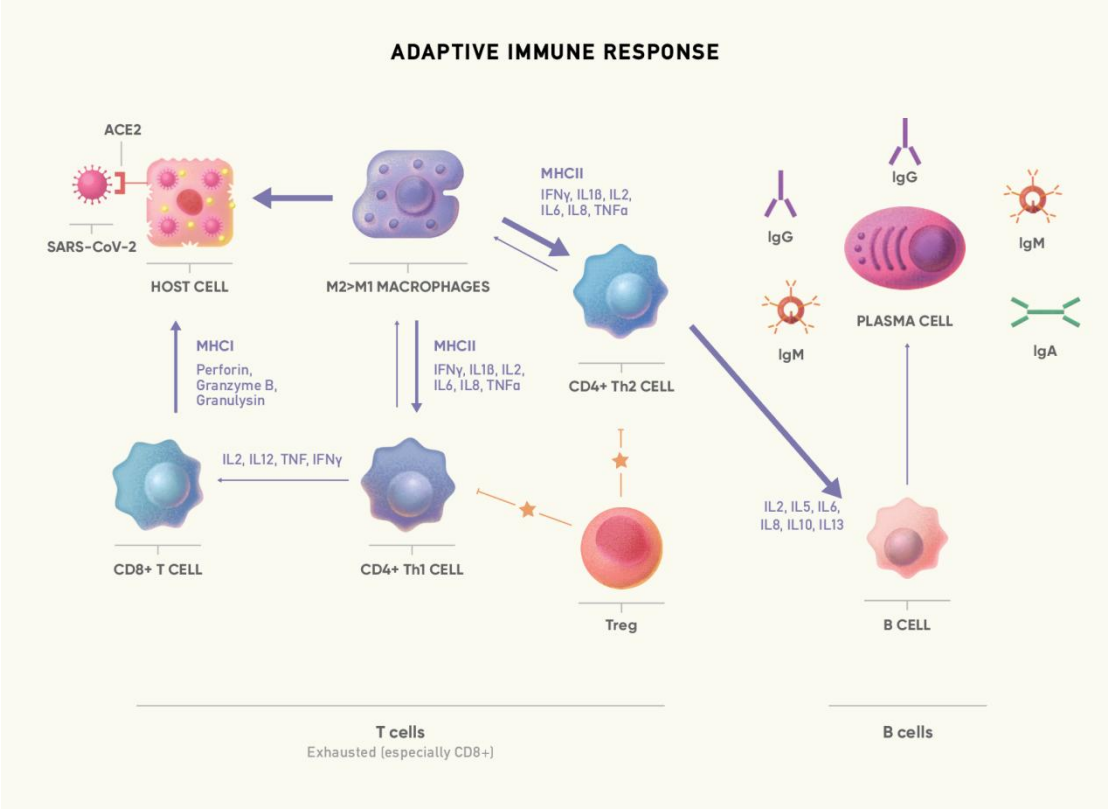


Figure 4. *The adaptive immune response against SARS-CoV-2 and its constituents. Adaptive immune responses are initially dampened on presentation in almost all patients irrespective of disease severity (as demonstrated by lymphopenia in blood tests on admission to hospital) [**Wang et al., 2020; Chiappelli et al., 2020**]. Cytokines, chemokines are depicted along with important immune receptors (MHCI-II). Widened arrows describe enhanced cell type-specific immune activation as demonstrated in severe/critical COVID-19 cases, in which adaptive responses are persistently dampened – qualitatively (T cells are exhausted) [**Zheng et al., 2020; Diao et al., 2020**] and quantitatively (failure of lymphocyte count to recover) [**Chen et al., 2020; Qin et al., 2020**]. Stars represent deregulated suppression on effector T cells by T-regulatory cells (Treg) whose levels are decreased [**Chen et al., 2020; Qin et al., 2020**]. Of note, predilection for M2 over M1 type of macrophage polarization with relatively increased B-cell compared to T-cell responses have been documented on necropsies [**Hanley et al., 2020**] (see arrows). For more information, see relative text.*

2.3 Immune response and SARS-CoV-2 severity

Epidemiological data from one of the largest cohort of patients in Hubei, China has shown that 20% of SARS-CoV-2 patients will have an unfavourable outcome, requiring oxygen therapy (15%) and critical care for organ support (5%) [**Epidemiology Working Group, 2020; Huang *et al.*, 2020; Xu *et al.*, 2020**]. The majority of these patients have underlying medical problems and/or are old (above the age of 60-65), which is an independent risk factor.

There is extensive research showing that clinical outcomes of patients with severe/critical COVID-19 is linked to an exaggerated immune response with adverse immune (immune exhaustion, possible immunosenescence) and clinical consequences [cytokine storm, Acute Respiratory Distress Syndrome (ARDS), Multi Organ Dysfunction Syndrome (MODS)], ultimately leading to death **(Figure3)** [**Huang *et al.*, 2020; Xu *et al.*, 2020; Wu *et al.*, 2020; Qin *et al.*, 2020; Mehta *et al.*, 2020; Suxin *et al.*, 2020; Li *et al.*, 2020; Mason *et al.*, 2020**].

Physiologically, the immune response is well orchestrated in time and space (spatiotemporally) such that its ultimate purpose - resolution of damage, repair, and healing – is fulfilled. Prerequisite to the latter is that its pro-inflammatory actions are counterbalanced with an equal and opposite anti-inflammatory wave of cytokines. For reasons still under investigation, this is not the case for 20% of COVID-19 patients. Their immune responses are out-of-sync leading to downstream immune deregulation which can take place early and/or late during the immunological cascade of events [**Catanzaro *et al.*, 2020**].

Following SARS-CoV-2 entry into host cells, IFNγ release – the earliest of immunological events - is key for robust antiviral innate immune responses that act as barrier to viral replication and dissemination at an early stage. In elderly patients with comorbidities this early response is delayed or severely diminished [**Yang *et al.*, 2020; O'Brien *et al.*, 2020; Blanco-Melo *et al.*, 2020; Gruber *et al.*, 2020; Trouillet-Assant *et al.*, 2020**] tipping the balance to an uncontrollable viral and subsequent cytokine dissemination, called cytokine storm (**Figure 3**). Low-grade chronic inflammation called inflammaging and other pre-existing conditions such as baseline immunosuppression, immune exhaustion, immunosenescence are additional factors that could affect this delicate balance [**Qin *et al.*, 2020; Bonafè *et al.*, 2020; Chiappelli *et al.*, 2020**].

A late event in the timeline of the immune response is antibody production by the humoral immune system. Both accelerated [**To *et al.*, 2020**] and delayed antibody responses [**Qu *et al.*, 2020**] have been linked to poor clinical course and higher disease severity in COVID-19 patients, further highlighting how late out-of-sync immune responses are associated with worsened outcomes.

Supportive to the above is the fact that the only drug so far proven to have a mortality benefit is dexamethasone (a corticosteroid drug with immune-inhibitory actions); it showed almost 33% reduction in mortality in severe (in need of oxygen therapy) and critically ill patients [**Ledford *et al.*, 2020; Villar *et al.*, 2020**].

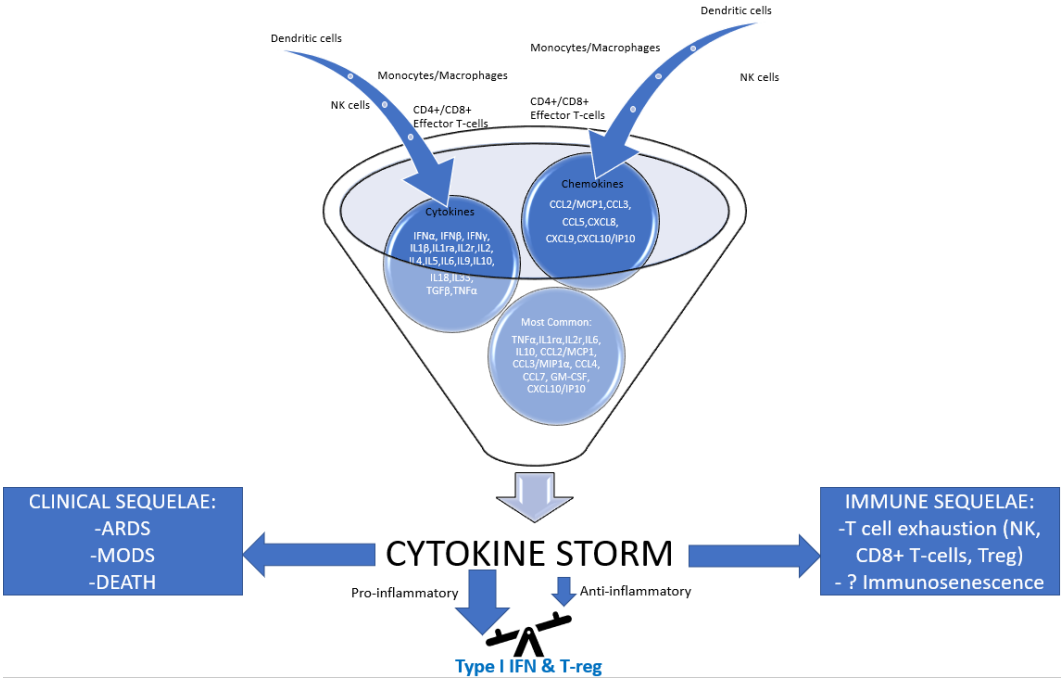


Figure 5. *Severe/critical disease outcomes for COVID-19 have been associated with an excessive immune response leading to cytokine storm which underpins unfavorable immune (right) and clinical (left) sequelae. Effector cells' input of cytokines and chemokines is depicted, while their most common profile can also be seen. In the foundation of cytokine storm lies an unbalanced pathological immune response with major pro- and little anti-inflammatory contribution secondary to at least in part, impaired IFN type I (early) and T-reg (late) actions [**Xiong et al., 2020; Chiappelli et al., 2020; Ye et al., 2020, Wang et al., 2020**]. See text for more information.*

2.4 The Timeline between SARS-CoV-2 infection and immunological response: Relationship between Viral Load, Clinical Symptoms/Picture and antibody response

Patients infected with the novel coronavirus will either develop symptoms or remain symptom-free. For those becoming symptomatic, there is a window before clinical symptoms arise - called incubation period - estimated to be 5-6 days on average, while it can be as short as 2 or as long as 14 days [**Lauer *et al.*, 2020**]. This is the evidence behind the 14-day length of quarantine in suspected individuals. With regards to asymptomatic individuals, their prevalence is still debatable in the literature [**Oyungerel Byambasuren *et al.*, 2020; Long *et al.*, 2020**].

Notably, antibodies have been detected in serum from patients irrespectively of their clinical picture (symptomatic vs asymptomatic). The kinetics of the viral load and the antibody response along with their temporal association has been studied and summarised in the diagram below (**Figure 4 diagram**).

On one hand, the viral load -detected through Reverse-Transcription Polymerase Chain Reaction (RT-PCR)- peaks at around the time of symptom onset [**He *et al.*, 2020; Zou *et al.*, 2020; Pan *et al.*, 2020; Kim *et al.*, 2020]** and then gradually declines towards the detection limit at about day 21, as antibody titres rise [**To *et al.*, 2020**].

Generally, antibodies -detected by immunodiagnostic/serological tests- are produced variably in time with most people seroconverting around the 2-week mark

(medial seroconversion interval around 13-15 days) (see **Table 1**). More specifically, antibody production seems to begin as early as 5-7 days post-symptom onset (p.s.o), then increase steadily with time reaching a point where almost all patients (95-100%) test positive within 21 days p.s.o. [**Long *et al.*, 2020; To *et al.*, 2020; Guo *et al.*, 2020; Xiang *et al.*, 2020; Okba *et al.*, 2020**]**.

Based on the above timescales, it is easy to understand experimental data demonstrating a decrease and increase in the positivity rate of PCR and serological antibody tests after week 1, respectively [**Zhao *et al.*, 2020; Guo *et al.*, 2020; Wölfel *et al.*, 2020**]** – results with important implications in diagnosis, contact tracing and pandemic surveillance.

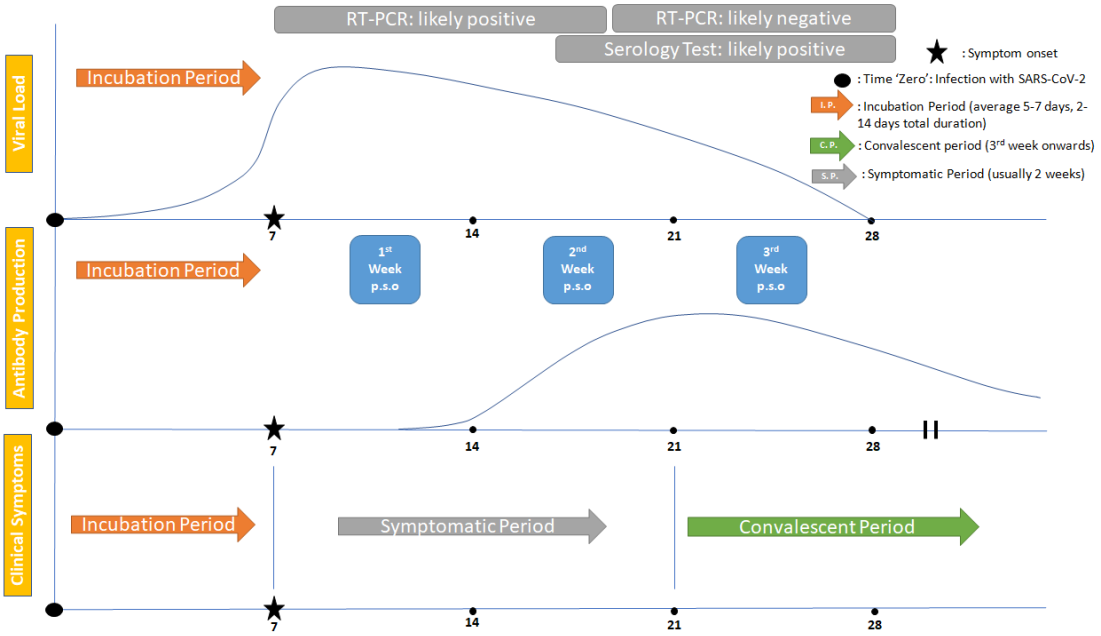


Diagram 1. *Antibody responses and viral load temporal kinetics as they correlate with clinical symptoms are depicted above. Estimated time intervals are based on data from several published studies. As shown in **Table 1** below, due to value variability, these should be considered approximations. During the incubation period, SARS-CoV-2 replicates silently in host cells without triggering IFN responses, leading to high viral loads upon symptom onset [**Blanco-Melo et al., 2020**]. Then, viral load steadily declines overtime while antibody responses become more pronounced over the following two to three weeks. During the first week post symptom onset (p.s.o), viral RNA is near its maximum value, rendering RT-PCR important for early diagnosis. In the third week p.s.o, antibody testing is more likely to aid in diagnosis. Finally, within the second week p.s.o, both tests could act complementary (as shown by boxes-overlap in the top) in equivocal clinical cases and help distinguish false negative RT-PCR results in patients admitted to the hospital late in their disease course; false negative RT-PCR results can occur as during the second and third week p.s.o (as depicted in the diagram above) viral RNA detected by RT-PCR decreases along with test's positivity rate [**Zhao et al., 2020; Guo et al., 2020; Wölfel et al., 2020**].*

Table 1. Non-exclusive table that contains studies measuring antibody responses against SARS-CoV-2 as a function of time.

Study (Reference)	Median Seroconversion (in days post-symptom onset)	Seroconversion interval for most patients (>90%, in days post symptom onset)
Long Q.X et al, 2020	13	within 19
To KK et al, 2020	10	within 14
Zao J et al, 2020	12	within 15
Wölfel R et al, 2020	10	within 14
OKBA NMA et al, medRxiv 2020	17	-
Liu W et al, 2020	15	within 18
Wei Yee Wan et al, medRxiv 2020	14	within 21

3. Considerations on Samples Collection and Virus Inactivation

3.1 Types of samples

A variety of samples have been used to document SARS-CoV-2 presence for diagnostic purposes. Although nasopharyngeal swab testing is the preferred sample for SARS-CoV-2 diagnosis along with oropharyngeal and recently saliva, samples from bronchoalveolar fluid, faeces, blood and urine specimens have also been tested to identify alternative routes of transmission [**Wang *et al.,* 2020**]. Urine specimens were always negative, while faecal specimens seem to remain positive even after upper airway (nasopharyngeal) samples had become negative [**Hindson *et al.,* 2020**].

3.2 Virus Inactivation

Similar to other coronaviruses, SARS-CoV-2 is sensitive to heat and ultraviolet radiation [**Cascella *et al.,* 2020**]. Additionally, these viruses can be effectively inactivated by lipid solvents including ethanol, chlorine-containing disinfectant, ether (75%), chloroform and peroxyacetic acid, except for chlorhexidine [**Cascella *et al.,* 2020**].

Although coronaviruses are generally thermolabile, SARS-CoV-2 appears to be stable at 37°C for at least 24 hours [**Wang *et al.,* MedRxiv 2020**]. Nevertheless, recently published data [**Kampf *et al.,* 2020; Wang *et al.*, 2020; Batéjat et al, 2020; Chin *et al.,* 2020**] demonstrate that patients' specimens can be heat treated to inactivate SARS-CoV-2, but certain conditions must pertain to preserve RNA integrity for further downstream nucleic acids amplification applications (NAATs).

Particularly, temperature treatment has been found to be related with specific incubation times in order to eliminate on one hand virus infectivity and on the other hand obtain maximal genome integrity. While various temperature vs incubation time combinations have been suggested as successful treatments, practically the following conditions have been mainly proposed and / or adopted [**Kampf *et al.,* 2020; Wang *et al.*, 2020; Batéjat *et al.,* 2020; Chin *et al.,* 2020**]:

1) 56° C for 30 min
2) 65° C for 15 min

Yet, there is some degree of disagreement. Samples with very low virus load, treated at 56° C for 30 min have been reported to exhibit an increased possibility to be scored as false negatives by RT-qPCR [**Pan *et al.,* 2020**]. Samples with high, medium and close to low viral load are not affected (see Figure 1 in Pan and collaborators [**Pan *et al.,* 2020**]). This finding appears to be not consistent with other reports [**Wang *et al.,* 2020**].

As an alternative to overcome this controversy, particularly in the case of swabs, is the implementation of transport media that contain acid guanidinium rather than the traditional universal transport medium. As demonstrated by Pan et al, [**Pan *et al.,* 2020**] they are superior for the following reasons:
1) infectivity is totally diminished once the swab-sample is submerged into the solution (no further treatments), thus no heat treatment is required
2) the sample vial, upon e.g. a chlorine spraying of the outside surface of the vial, is safe for further handling

3) RNA integrity is best preserved for molecular analyses (RT-PCR and sequencing) allowing detection of low copies virus in patients' samples.

3.3 General safety measures

Safety measures include a variety of aspects: 1) proper packaging and transport of samples, 2) safety rooms, ideally possessing negative pressure and UV irradiation, equipped with Class II or higher safety cabinets with dedicated equipment, such as centrifuge(s), freezer(s) for sample storage, water baths, pipettes, racks, waste disposal and consumables / reagents, often designated dirty room, for RNA extraction, 3) a clean room, furnished also with the necessary equipment and dedicated consumables / reagents and UV irradiation, for performing molecular analysis (set up RT-qPCR, sequencing reactions), 4) detailed training for operators on safety measures (wearing gloves, mask and appropriate lab coat), decontamination measures, sample handling and waste disposal.

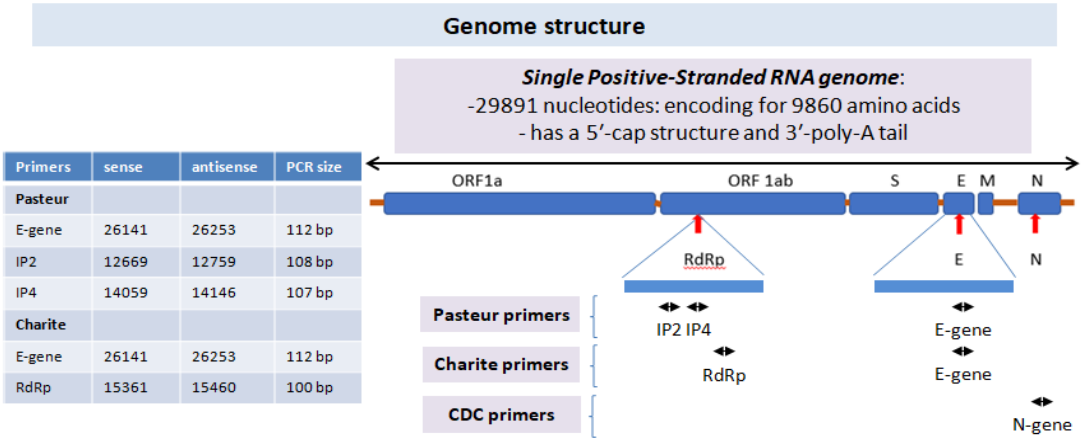
4. Development of "In House" Molecular Methods

4.1 Molecular methods for detecting Sars-CoV-2

Development of reliable diagnostic assays is crucial for early detection of SARS-CoV-2 and thus reduction of transmission and the basic reproductive number (R0). The gold standard for COVID-19 diagnosis is based on the detection of unique sequences of viral RNA by Nucleic Acid Amplification Testing (NAAT), such as real-time Reverse Transcription-Polymerase Chain Reaction (rRT-qPCR) with confirmation by nucleic acid sequencing assays when necessary, on respiratory specimens [**WHO laboratory testing 2020**].

Several commercial RT-PCR based SARS-CoV-2 viral detection methods are currently available by many vendors. Most of these methods target the viral genes N, S, E and RdRp. The adopted algorithm for laboratory confirmation of SARS-CoV-2 infection requires a positive RT-PCR result for at least two different targets on the virus genome, one of which is specific for COVID-19 [**WHO laboratory testing 2020**].

Among the most widely used RT-PCR assays are the Institut Pasteur (Paris France), Charite (Berlin Germany), US Centers for Disease Control and Prevention (CDC United States) and China CDC (China). Each of them targets different regions in the virus genome **(Figure 6**).



Primers	sense	antisense	PCR size
Pasteur			
E-gene	26141	26253	112 bp
IP2	12669	12759	108 bp
IP4	14059	14146	107 bp
Charite			
E-gene	26141	26253	112 bp
RdRp	15361	15460	100 bp

Figure 6. *The three most popular primers used for SARS-CoV-2 detection and their amplification targets in the genome of the virus.*

The relative performance of some laboratory RT-PCR techniques has been recently reported [**Jung *et al.*, 2020; Vogels *et al.*, 2020, Etievant *et al.*, 2020**]. All RT-PCR assays performed well for SARS-CoV-2 detection. The most sensitive assays were the RdRp Institut Pasteur (IP2, IP4), N China CDC and N1 US CDC, while N Charite and N2 US CDC lacked specificity [**Vogels *et al.*, 2020; Etievant *et al.*, 2020**].

The majority of RT-PCR assays for SARS-CoV-2 detection are based on conversion of a specific target of the viral RNA to cDNA by reverse transcription, amplification and detection of the cDNA with sequence-specific primers and DNA probes labeled with a fluorescent molecule and a quencher molecule. These reactions are applied as a "one-step procedure", performing the reverse transcription of viral RNA and amplification steps sequentially in a single vial. The one-step application provides fast results and minimize the risk of cross contamination between the two

enzymatic reactions [**Carter et al 2020**]. Nevertheless, the one-step RT-PCR is less sensitive compared to the two-step RT-PCR, in which reverse transcription and amplification are performed separately [**Wong & Medrano 2005**]. Therefore, an "in house" "two-step RT-PCR" could increase the assay sensitivity.

All available assays for SARS-CoV-2 detection define positive and negative samples according to cycle threshold (Ct) values from qualitative rRT-PCR [**https://www.ecdc.europa.eu/en/all-topics-z/coronavirus/threats-and-outbreaks/covid-19/laboratory-support/questions, US CDC 2020**]. Nevertheless, Ct values can easily be affected by batch effect, thus all runs must be carefully monitored [**Han *et al*., 2020**]. We propose a more reliable alternative, unaffected by such confounding factors, based on the quantitation of unknown samples using as a reference point a standard curve in each rRT-PCR run. The latter can be plotted by preparing and analyzing serial dilutions of a positive control, such as plasmids of known copy numbers.

Recently some preliminary studies correlate viral load with symptom onset, disease severity and different types of clinical specimens [**Zou *et al*., 2020, Lescure *et al*., 2020, Pan *et al*., 2020; Han *et al*., 2020**]. Absolute quantitation of SARS-CoV-2 with real time quantitative PCR (qPCR) could subscribe valuable information to clinicians for determining possible correlations between viral load and virus transmission dynamics, disease outcome and possible therapeutic interventions.

During COVID-19 pandemic, the need for immediate testing and the lack or delay in the delivery of commercial kits due to increased global demand, points out the emergency for development of "in house" protocols for RNA extraction and rRT-qPCR capable to detect and quantify SARS-CoV-2. As an alternative, we established a conventional RNA extraction and a quantitate two-step real time PCR protocol for SARS-CoV-2 detection (**Figure 7**).

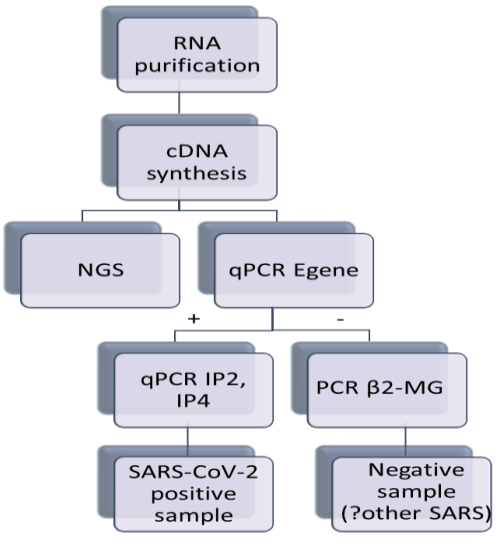


Figure 7. *Workflow diagram for SARS-CoV-2 detection with "in house" NAAT.*

4.2 RNA purification

4.2.1 The Acid Guanidinium Phenol Chloroform (AGPC) method of extraction

The best-known RNA isolation method, on which rely many commercial kits, is the "*Acid Guanidinium Phenol Chloroform (AGPC) extraction method*", first described by Chomczynski and Sacchi [**Chomczynski & Sacchi, 1987**]. In AGPC, guanidinium thiocyanate is employed as a chaotropic agent, due to its strong denaturating capability. Addition of acid phenol and chloroform ensures the separation of RNA from DNA and proteins. Specifically, DNA and proteins precipitate in the organic phase, while RNA remains in the upper aqueous phase, from which it can subsequently be precipitated. An updated version of this protocol has been successfully applied in our lab.

RNA extracted through this protocol must be validated by spectrophotometric analysis (estimation of the 260/280 nm ratio) and real time PCR amplification of a reporter gene, such as β-microglobulin or others.

The RNA extraction protocol is as follows:

1) Following viral inactivation (see section *3.2*), vortex swabs for 3 min
2) Add 1 mL of solution D to 0.25 mL of sample in a sterile 1,5-ml eppendorf tube and mix by pipetting.
3) To the above solution add the following sequentially:
    a) 0.1 ml of 2 M sodium acetate, pH 4.0, mix thoroughly by inversion;
    b) 1 ml water-saturated phenol, mix thoroughly by inversion;
    c) 0.2 ml of chloroform/isoamyl alcohol (49:1), shake vigorously by hand for 10 s.
4) Cool samples on ice for 15 min.
5) Centrifuge for 20 min at 10,000g at 4° C.
6) Transfer the upper aqueous phase to a clean tube.
7) Add 5–10 μg of RNase-free glycogen or 10-20 μg/ml tRNA as a carrier to the aqueous phase (Optional).
8) Add to the aqueous phase 1 ml isopropanol to precipitate RNA.
9) Incubate samples for at least 1h, at –20° C.
10) Centrifuge for 20 min at 10,000g at 4° C and discard the supernatant.
11) Resuspend RNA pellet in 0.5ml of 75% ethanol and mix by vortexing for a few seconds.
12) Incubate samples for 10–15 min at room temperature to dissolve possible residual traces of guanidinium.
13) Centrifuge for 5 min at 10,000g at 4° C and discard the supernatant.
14) Repeat steps 11-13.
15) Air-dry the RNA pellet for 5–10 min at room temperature.
16) Dissolve RNA pellet in 100–200μl of either DEPC-treated water or 0.5% SDS.
17) Incubate RNA for 10–15 min at 60° C to ensure complete solubilization.
18) Store extracted RNA at -80° C.

For solutions preparation see Appendix 1.

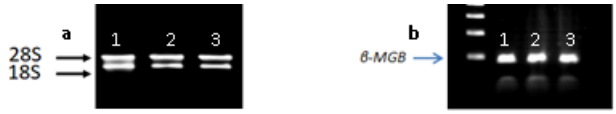


Figure 8. *Comparison of the RNA extraction efficiency and quality in A549 cells by: (a) gel electrophoresis, b) Conventional RT-PCR amplification of β-microglobulin (β-MGB). (lane 1 - AGPC method, lane 2 - NucleoZOL reagent, lane 3 - TRIzol™ reagent).*

4.2.2 Application of AGPC method on clinical samples

AGPC method was applied in cell lines and nasopharyngeal swabs and its performance was compared with other similar commercially available RNA extraction methods such as TRIzol™ reagent (PanReac Applichem A4051) and NucleoZOL (Macherey Nagel). RNA extraction efficiency and quality were assessed by gel electrophoresis and real time RT-PCR amplification of β-microglobulin (β-MGB) as a reporter gene on a RotorGene6000 thermocycler (**Figure 8, 9**).

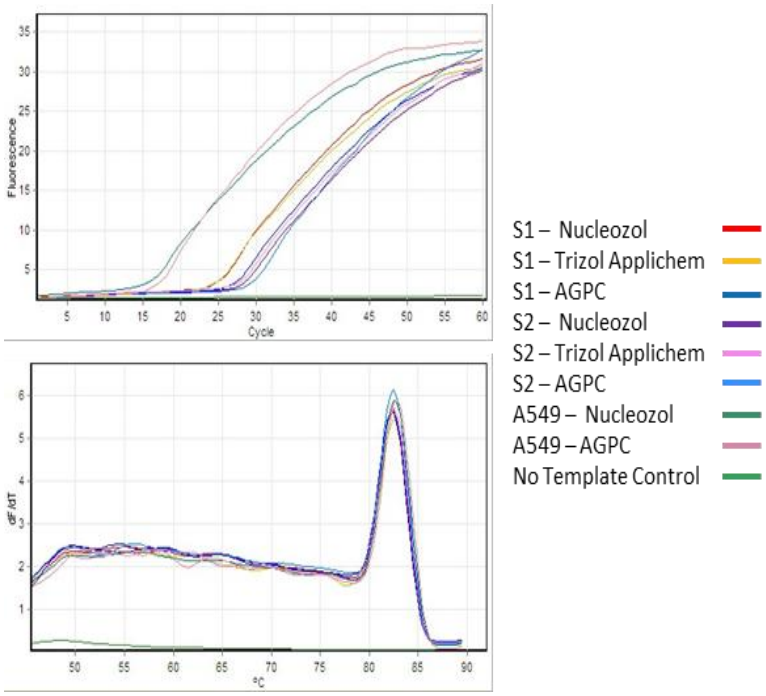


Figure 9. *Comparison of the RNA extraction efficiency and quality in nasopharyngeal swabs with NucleoZOL reagent, TRIzol™ reagent and AGPC method with real time PCR for β-MGB.*

AGPC RNA purification methodology yields compatible results with other commercial methods and can be employed for SARS-CoV-2 detection.

4.3 Real time Reverse Transcription quantitative Polymerase Chain Reaction (rRT-qPCR)

4.3.1 One-Step Real time Reverse Transcription quantitative Polymerase Chain Reaction (rRT-qPCR)

Based on the published protocol of the Institut Pasteur for SARS-CoV-2 detection we employed the one-step method [**https://www.who.int/emergencies/diseases/novel - coronavirus -2019 / technical-guidance/laboratory - guidance**]. The suggested algorithm according to Corman et al. is based on the amplification of a conserved region of the E gene – used as a confirmatory assay of coronavirus presence in the examined sample - and subsequent amplification of two RdRp gene targets, IP2 and IP4, to confirm the identity of SARS-CoV-2 [**Corman *et al.*, 2020**].

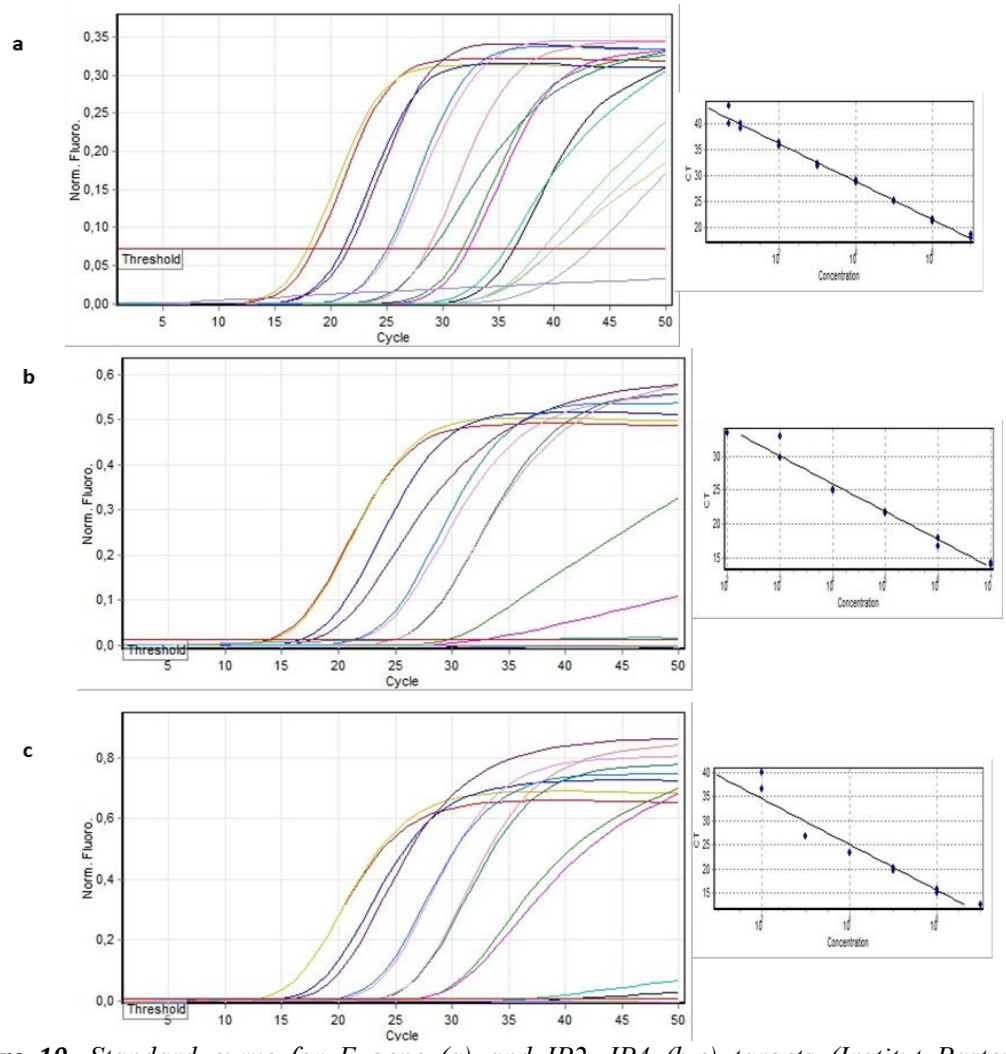


Figure 10. *Standard curve for E gene (a) and IP2, IP4 (b,c) targets (Institut Pasteur's protocol)*

According to Institut Pasteur's one-step RT-PCR protocol we constructed a standard curve using the provided positive control for real time RT-PCR, an *in vitro* transcribed RNA derived from the strain BetaCoV_Wuhan_WIV04_2019 (EPI_ISL_402124), which contains regions of the RdRp and E genes as positive

strands on a RotorGene 6000 [**https://www.who.int/docs/default-source/coronaviruse/real-time-rt-pcr-assays-for-the-detection-of-sars-cov-2-institut-pasteur-paris.pdf?sfvrsn= 3662fcb6_2**] (**Figure 10**).

Application of the Institut Pasteur's protocol on the RotorGene 6000 demonstrated increased sensitivity for E gene amplification and decreased sensitivity for IP2 and IP4 targets, as it was assessed by determination of the Limit of Detection (LoD). LoD is defined as the target's lowest concentration that can be detected in ≥95% of repeated measurements. LoD for E gene was estimated as 7.7 copies/reaction at a statistical significance level of 0.05. A linear regression model fits our RT-PCR experimental data as depicted in the estimated correlation coefficient value (R-Square –R^2) (**Figure 11**).

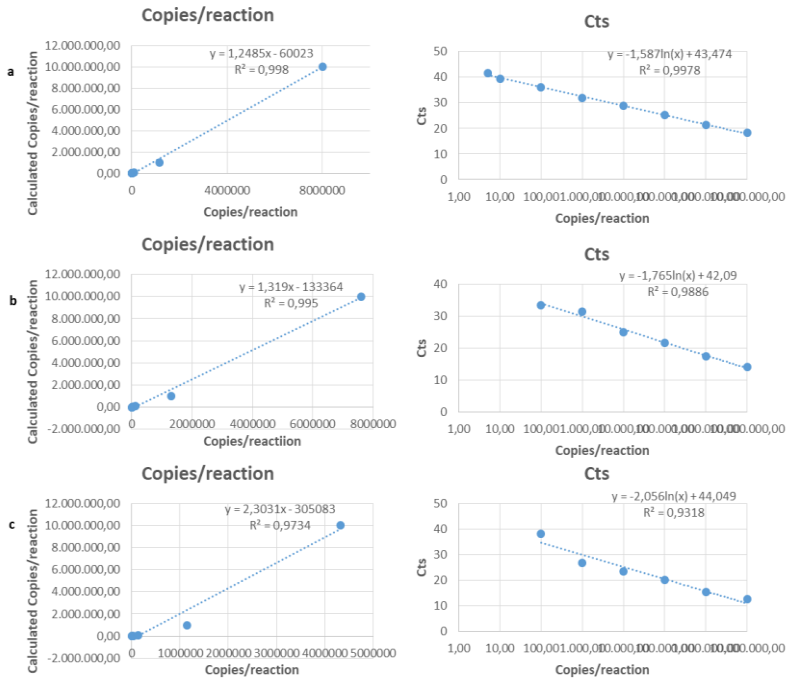


Figure 11. *Estimated LoD for E gene (a) and IP2, IP4 (b,c) targets (Institut Pasteur's protocol)*

4.3.2 Two-Step Real time Reverse Transcription quantitative Polymerase Chain Reaction (rRT qPCR)

We applied a 'two-step" qPCR protocol with serial dilutions of Institut Pasteur's *in vitro* transcribed SARS-CoV-2 RNA positive control and tested the sensitivity by defining LoD with singleplex qPCR for E gene and multiplex for IP2, IP4 targets.

4.3.2.1 cDNA synthesis

cDNA synthesis of serial dilutions of Institut Pasteur's *in vitro* transcribed RNA positive control was done according to following procedure:

1) Prepare cDNA synthesis reaction with LunaScript RT SuperMix (E3010) by mixing:

a) LunaScript RT SuperMix 4µl
b) Serial dilutions of Institut Pasteur's in vitro transcribed RNA positive control 10µl
c) RNAse-free water up to 20µl final volume

2) Incubate reactions in a RotorGene6000 thermocycler with the following setup:

a) Primer Annealing: 25° C for 2 min
b) cDNA synthesis: 55° C for 10 min
c) Heat Inactivation: 95° C for 1 min

4.3.2.2 qPCR

Singleplex qPCR for E gene and multiplex for IP2, IP4 targets was performed as follows.

1) Prepare qPCR reaction for E gene with Luna Universal Probe qPCR Master Mix (NEB #M3004) by mixing:

a) Luna Universal Probe qPCR Master Mix 10µl
b) BSA 1µl
c) E gene Forward primer 0.8µl (10µM)
d) E gene Reverse primer 0.8µl (10µM)
e) E gene Probe 0.4µl (10µM)
f) cDNA products 1µl
g) RNAse-free water up to 20µl final volume

2) Incubate reactions in a RotorGene6000 thermocycler with the following setup:

a) Initial denaturation: 95° C for 1 min, 1 cycle
b) Denaturation: 95° C for 15 seconds 45 cycles
Extension: 58.5° C for 30 seconds

3) Prepare qPCR reaction for IP2 and IP4 targets with Luna Universal Probe qPCR Master Mix (NEB #M3004) by mixing:

a) Luna Universal Probe qPCR Master Mix 10µl
b) BSA 1µl
c) IP2 Forward primer 0.8µl (10µM)
d) IP2 Reverse primer 0.8µl (10µM)
e) IP4 Forward primer 0.8µl (10µM)
f) IP4 Reverse primer 0.8µl (10µM)
g) IP2 Probe 0.3µl (10µM)
h) IP4 Probe 0.3µl (10µM)
i) cDNA products 1µl
j) RNAse-free water up to 20µl final volume

4) Incubate reactions in a RotorGene6000 thermocycler with the following setup:

a) Initial denaturation: 95° C for 1 min, 1 cycle
b) Denaturation: 95° C for 15 seconds 45 cycles

Extension: 58° C for 30 seconds

The in house “two-step” protocol displays equal sensitivity with Institut Pasteur’s “one-step” protocol, for E gene and IP2, IP4 targets, in concordance with the general assumption and the estimated LoDs (**Figures 12, 13**).

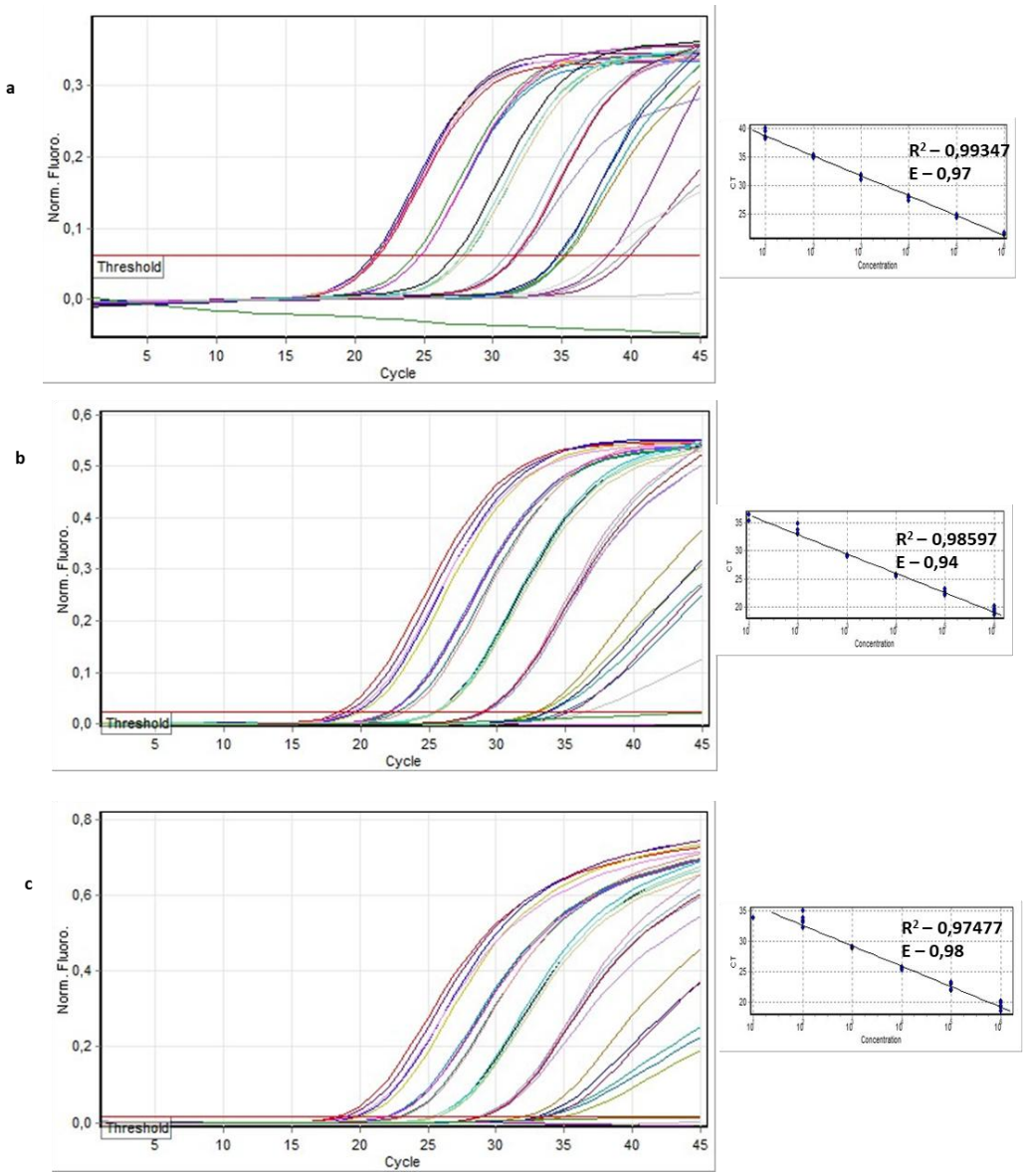


Figure 12. *Two-step “in-house” qPCR standard curve for E gene (a), IP2 (b) and IP4 (c) targets.*

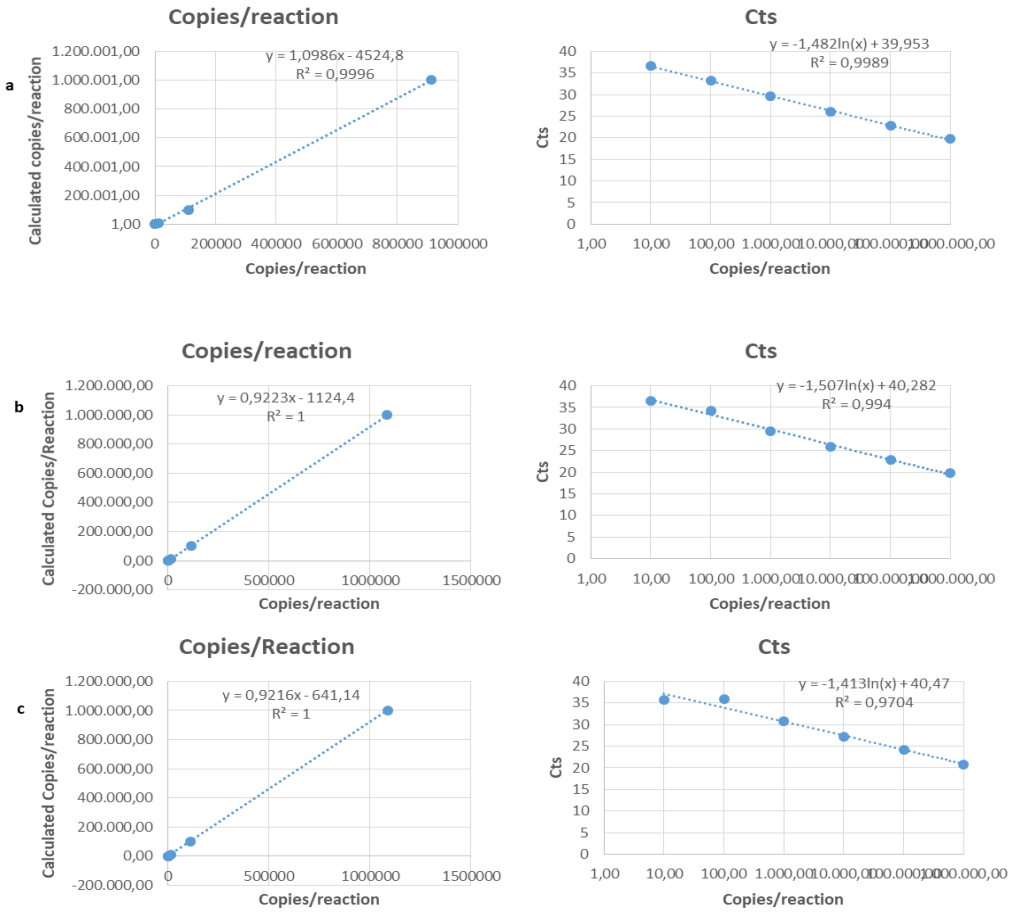


Figure 13. *Standard curve for E gene (a) and IP2, IP4 (b,c) targets (Institut Pasteur's protocol)*

4.4 Application of the "two-step" qPCR protocol on clinical samples

36 clinical nasopharyngeal swab samples obtained from "ATTIKO" University Hospital in Athens were tested. Results showed 20 to be positive and 16 to be negative. Representative cases are shown below.

RNA was extracted using the AGPC extraction method. cDNA synthesis was performed with LunaScript RT SuperMix. Different RNA concentrations were tested. 250 ng RNA were found sufficient for cDNA synthesis and efficient downstream NAAT applications (**Figure 14**).

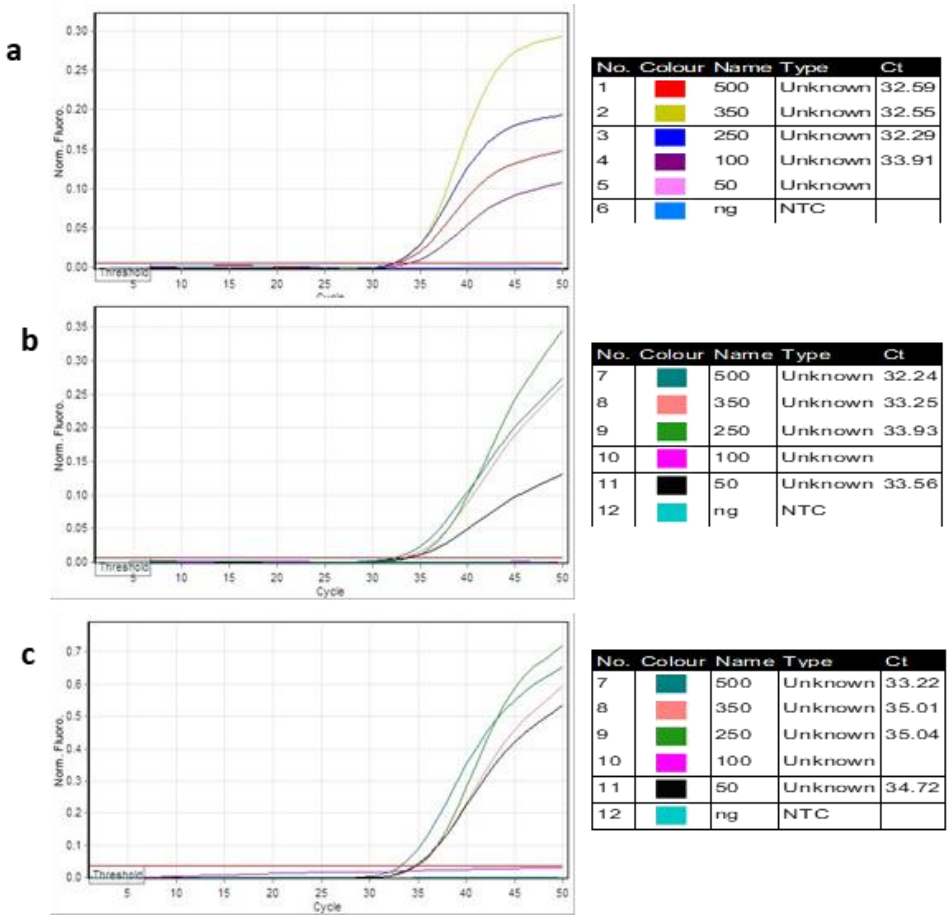


Figure 14. *Different RNA concentrations for cDNA synthesis [E gene (a), IP2(b) and IP4 (c) targets].*

cDNA products from clinical samples were amplified using qPCR for E gene and IP2, IP4 targets. In each qPCR reaction, cDNA products of serial dilutions of Institut Pasteur's *in vitro* transcribed RNA positive control were used as standards. For sample viral load quantitation, a range of 10-10^3 viral copies/reaction were used (**Figure 15**).

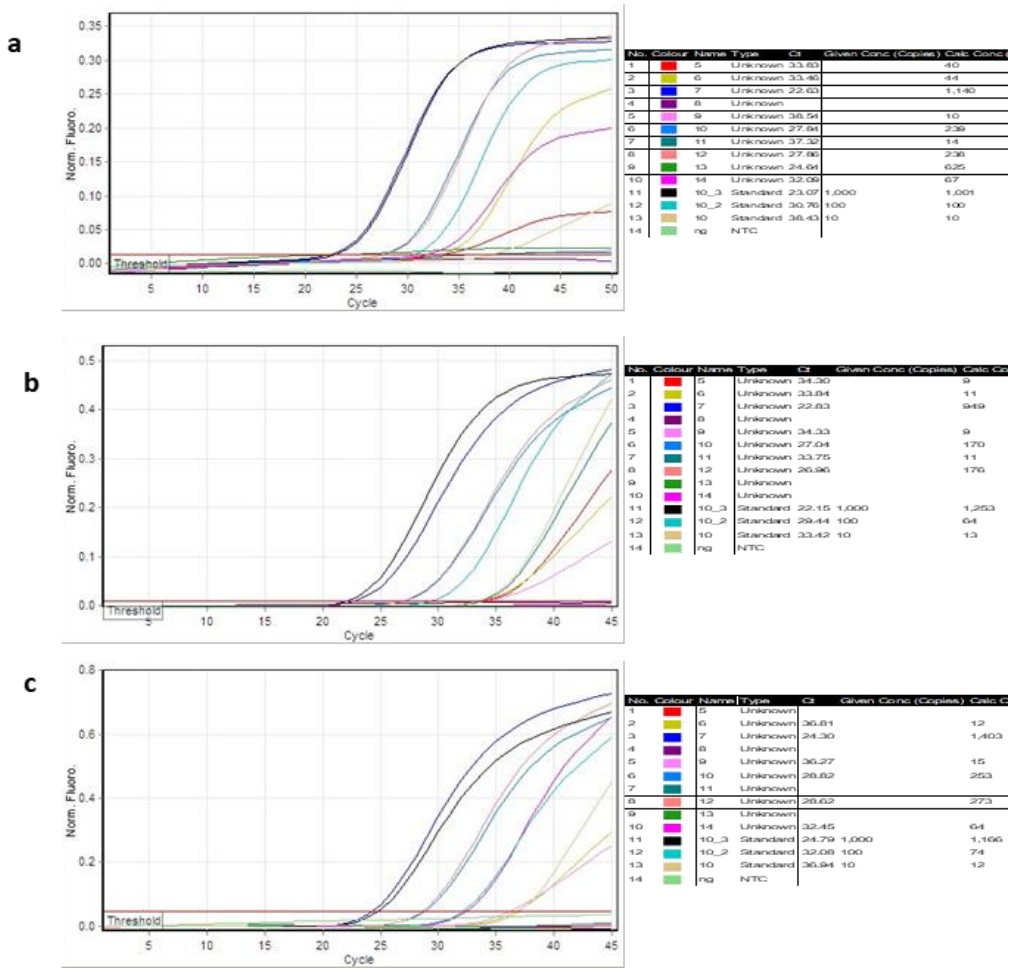


Figure 15. *qPCR for E gene (a), IP2(b) and IP4 (c) targets in representative clinical samples.*

4.5 Application of other molecular assays for detecting SARS-CoV-2

As mentioned above, the relative performance of some laboratory rRT-qPCR techniques has been published recently [**Jung *et al.*, 2020; Vogels *et al.*, 2020; Etievant *et al.*, 2020**]. The most sensitive assays were the RdRp Institut Pasteur (IP2, IP4), N China CDC and N1 US CDC, while N Charite and N2 US CDC lacked specificity [**Vogels *et al.*, 2020; Etievant *et al.*, 2020**].

4.5.1 US CDC assay

We applied US CDC protocol in clinical nasopharyngeal swab samples positive for SARS-CoV-2, which were obtained from "ATTIKO" University Hospital in Athens. US CDC recommends 'one-step' rRT-qPCR targeting three independent regions of the N-gene and amplification of RNAse P (RP) as a reporter-gene. We performed the proposed protocol as per instructions, with the exception of the rRT-qPCR enzyme mastermix. Instead of TaqPath™ 1-Step RT-qPCR Master Mix that is suggested by US CDC, we used SuperScript™ III Platinum™ One-Step qRT-PCR Kit (11732020) and SARS-CoV-2 diagnosis was confirmed (**Figure 16**).

Singleplex rRT-PCR for N1, N2, N3 targets and RP reporter-gene was done according to following procedure:

1) Prepare rRT-qPCR mastermix with SuperScript™ III Platinum™ One-Step qRT-PCR Kit for N1, N2 and N3 targets by mixing:

a) 2X Reaction Mix 12.5µl
b) $MgSO_4$ 0.4µl
c) BSA 1µl
d) Combined primer/probe mix (N1, N2, N3, RP 1.5µl)
e) SuperScrip III RT/Platinum Taq mix 1µl
f) RNA 5µl
g) RNAse-free water up to 25µl final volume

2) Incubate reactions in a RotorGene6000 thermocycler with the following setup:

a) Reverse Transcription 55° C for 20 min 1 cycle
b) Initial denaturation: 95° C for 3 min 1 cycle
c) Denaturation: 95° C for 15 seconds 45 cycles
Extension: 55° C for 30 seconds
d) Cooling: 40° C for 30 seconds 1 cycle

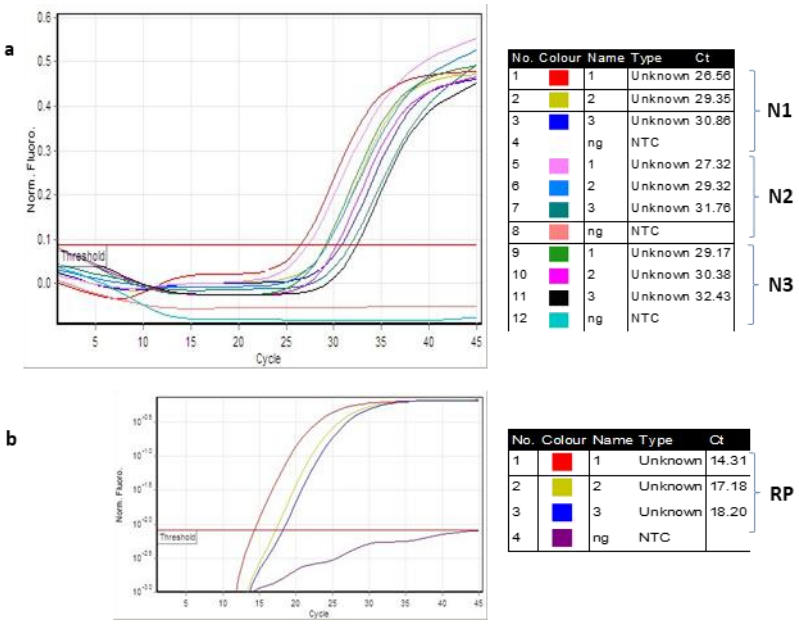


Figure 16. *Application of the CDC protocol in clinical samples scoring positive for SARS-CoV-2.*

4.5.2 Charite assay

The first published rRT-qPCR assay developed and listed in WHO is from Charite, Berlin. This assay was initially adapted for SARS-related CoVs and later modified for SARS-CoV-2 detection at the onset of COVID-19 pandemic. The Charite assay

recommends E gene amplification as a pan-coronavirus test, followed by confirmation of SARS-CoV-2 presence by targeting RdRp gene [**Corman *et al.*, 2020].**

We applied Charite assay according to its instructions in clinical nasopharyngeal swab samples positive for SARS-CoV-2, which were obtained from "ATTIKO" University Hospital in Athens. Improved efficiency in RdRp assay was observed by increasing annealing temperature at 58.5^{o} C. Amplification of No Template Control (NTC - water) was noticed in most experiments, which could be attributed to either primer contamination or nonspecific amplification, as reported elsewhere [**Etievant *et al.*, 2020**] (**Figure 17**).

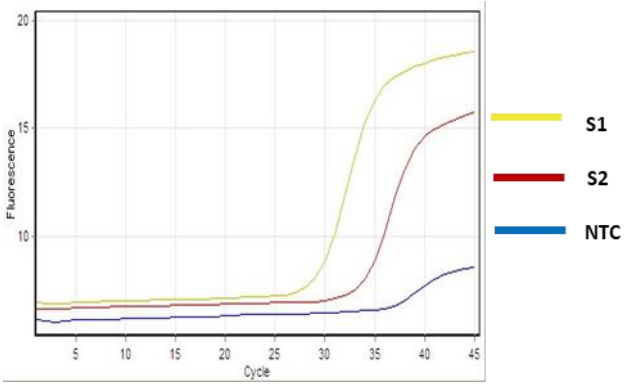


Figure 17. *Application of the Charite RdRp assay in clinical samples (S1-S2 - clinical samples, NTC- No Template Control).*

It has been shown that the RdRp reverse primer contains an incorrect degenerate base (S). Additionally, nucleotide replacements in RdRp probe have also been proposed for Charite assay improved sensitivity [**Pillonel *et al.*, 2020; Etievant *et al.*, 2020**]. We believe that through such improvements, application of a two-step procedure in Charite assay would be of great importance.

4.6 Sequencing methods for detecting Sars-CoV-2

4.6.1 Ion Torrent based protocol for sequencing the whole genome of Sars-CoV-2

One of the first Next Generation Sequencing (NGS) platforms developed for specific targeting the SARS-CoV-2 genome employed the Ion Torrent technology. One of the advantages of this platform is the highly specific Ion AmpliSeq SARS-CoV-2 Research Panel that enables interrogation of the complete SARS-CoV-2 genome for epidemiological research. The protocol can be roughly divided in the following subsections (**Figure 18**):

1) Substrate Material: RNA extracted from nasopharyngeal samples, sputum or any other bioptic material, previously characterized by RT-qPCR for high viral load.

2) Reverse Transcription: extracted RNA from previous step is reverse transcribed with the SuperScript™ IV VILO™ Master Mix (Cat No 11766050, Thermo Fisher Scientific).

3) AMPLISEQ Library Construction: for the generation of the cDNA library the guidelines of the protocol MAN0017003 are followed using the reagents included in the Ion AmpliSeq™ Library Kit Plus (Thermo Fisher Scientific).

Specifically, the cDNA is amplified with a highly multiplexed set of primers that covers the whole genome of SARS-CoV-2 (SARS COV 2 Ampliseq Panel). This set includes 247 pairs of PCR primers that are specific for virus genome. As a quality feature, an internal control consisting of primer pairs for 5 human mRNA (reference) gene targets that are included in every sample to monitor for errors. The panel covers overall >99% of the ~30Kb viral genome. The amplification process is amenable for concurrent handling of high numbers of samples and for this it can employ PCR plates with 96 wells compatible with all conventional cyclers available in every laboratory. Multiple such 96 reactions (corresponding to 96 samples) can be run concurrently.

Amplicons generated are incubated with the FuPa reagent to digest the primers and phosphorylate the amplicons for adapter ligation using IonXpress Barcodes. The use of Barcode adaptors in the library preparation facilitates the simultaneous sequencing of multiple libraries, providing economy in reagents usage and work time. The products from the ligase reaction are purified with Ampure XP magnetic beads and equilibrated with the Ion Equalizer kit (Thermo Fisher Scientific). Alternatively, the molecular concentration of the library is defined with fluorometry (Qubit fluorometer - Thermo Fisher Scientific). In the latter case the quality control is simplified and faster, without the need for capillary electrophoresis, thus reducing the cost.

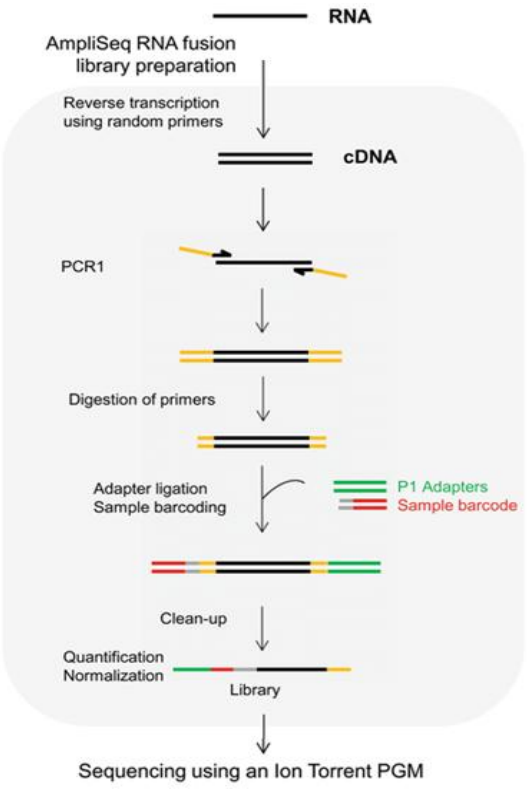


Figure 18. *Schematic presentation of the flow diagram of the Ion Torrent based protocol for sequencing the whole genome of Sars-CoV-2*

4) Samples preparation for sequencing reaction: the generated libraries are mixed at equimolar level and can include up to 80 libraries/sequencing reaction using the Ion CHEF apparatus. The procedure running on the Ion CHEF is fully automated, including upload on the sequencing chip (Ion 540).

The sequencing reaction is performed on the Ion S5 with an estimated running time of 2 hours. From each 540 chip >80 million reads (corresponding to an estimated volume of data >1 million read/sample) securing an in-depth read and increased sensitivity.

5) Bioinformatic analysis of sequencing results: for the bioinformatic analysis and to secure simplified, non-expert usage and easy interpretation of the data without employing super-computers the software SARS-COV-2 Research Plug-in Package (Thermo Fisher Scientific) is freely available for the analysis of the samples performed with the Ampliseq SARS-COV2 panel. The plugin includes: i) specialized algorithm for polymorphism detection allowing the usage of the variant calling in order to compare with published reference sequences, ii) IRMAreport packages that facilitate the generation of consensus sequences from viral subtypes that exhibit high mutagenesis, iii) the AssemblerTrinity package that performs *de novo* assembly of the viral genomes. All data produced are in the FASTQ and BAM format and are thus compatible with other available bioinformatics software (**Figure 19**). All plugins are part of the Ion Torrent Suite™ Software (Thermo Fisher Scientific).

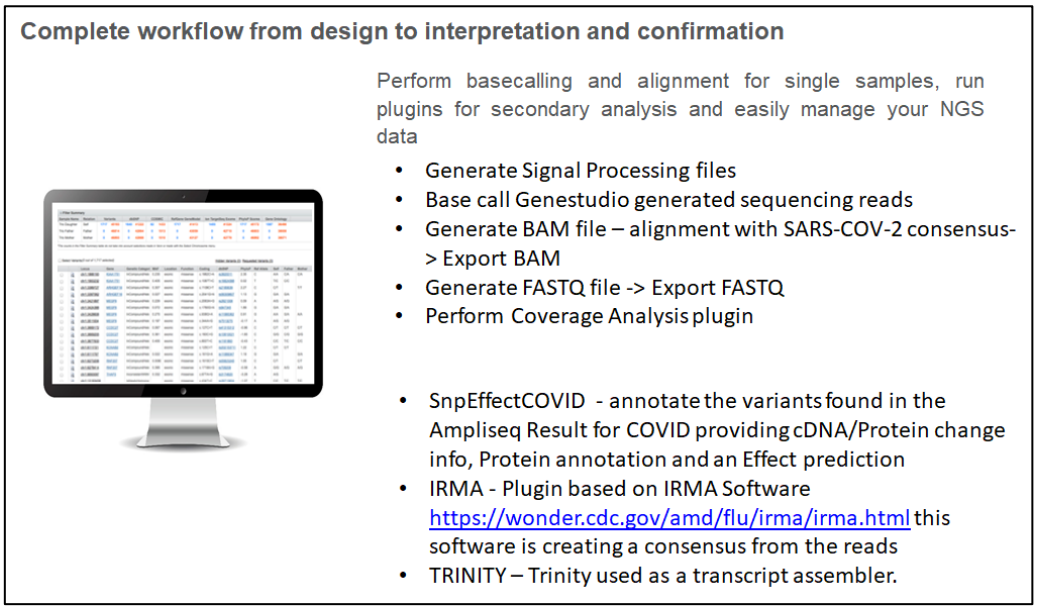


Figure 19. *Ion Torrent Suite™ Software for sequencing data analysis.*

4.6.2 Application of the Ion Torrent protocol for sequencing the whole genome of Sars-CoV-2 in clinical samples

Samples with high viral load, determined by RT-qPCR, were subjected to reverse transcription with the SuperScript™ IV VILO™ Master Mix (Cat No 11766050, Thermo Fisher Scientific) following the manufacturer's instruction (**Figure 18**). The generated cDNAs were subjected to libraries formation for each sample as described in the previous section. After quality control, the libraries were uploaded on a 540

chip and finally sequencing was performed. The Ion Torrent Suite™ Software (**Figure 19**) was used to analyse the obtained data. Following a quality control of the NGS data (**Figure 20**), FASTQ files were obtained for each sample and subjected to BLASTn. Homology search using the Wuhan SARS-CoV-2 as a reference gene demonstrated detection of new variants of the virus in the Greek population (**Figure 21**).

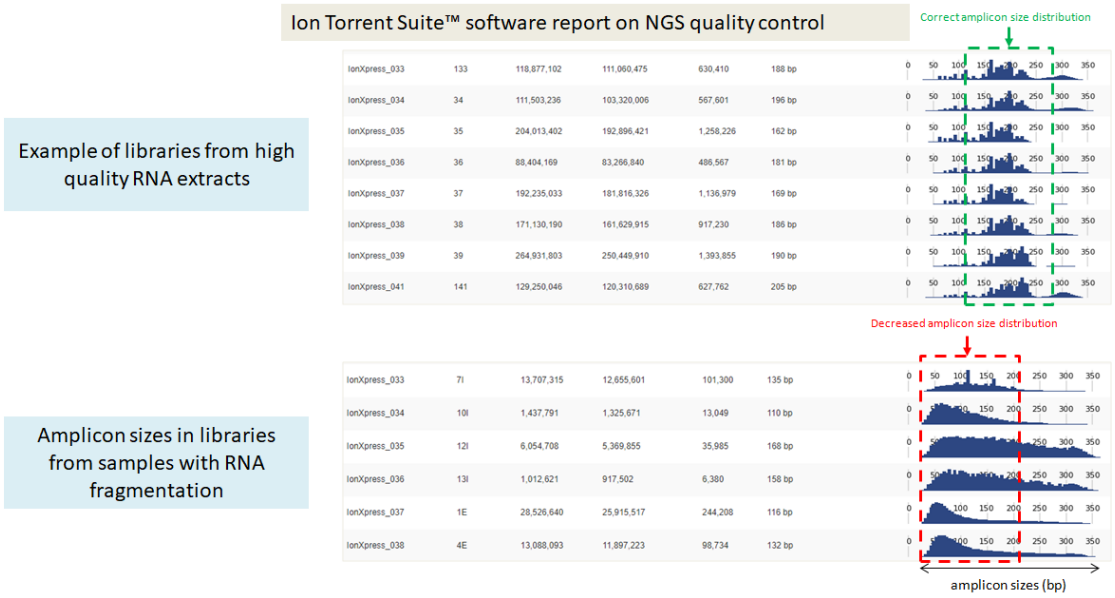


Figure 20. *Ion Torrent Suite™ software report on NGS quality control of examined samples.*

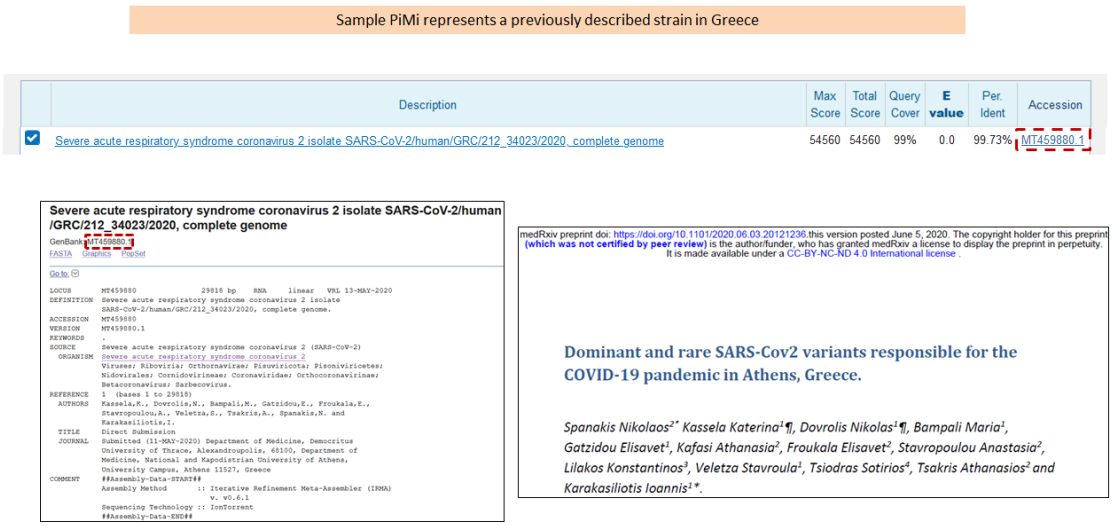


Figure 21. *Representative BLASTn search showing detection of a new SARS-CoV-2 strain that is homologous to a recently described strain in Greece.*

5. Development of "in house" immunological methods

5.1 ELISA methods for detecting Sars-CoV-2

Enzyme linked immunosorbent assay (ELISA) is one of the most common immunological method in order to accurately detect the presence of an infectious agent in biological fluids. There are several different methods, however, there are two different methods that are most suitable for the detection of viral particles in patient samples: Antigen Down ELISA and Double Antigen bridging assay (**Figure 22**). An assessment was performed in order to determine the immunoassay that exhibits the highest specificity and sensitivity.

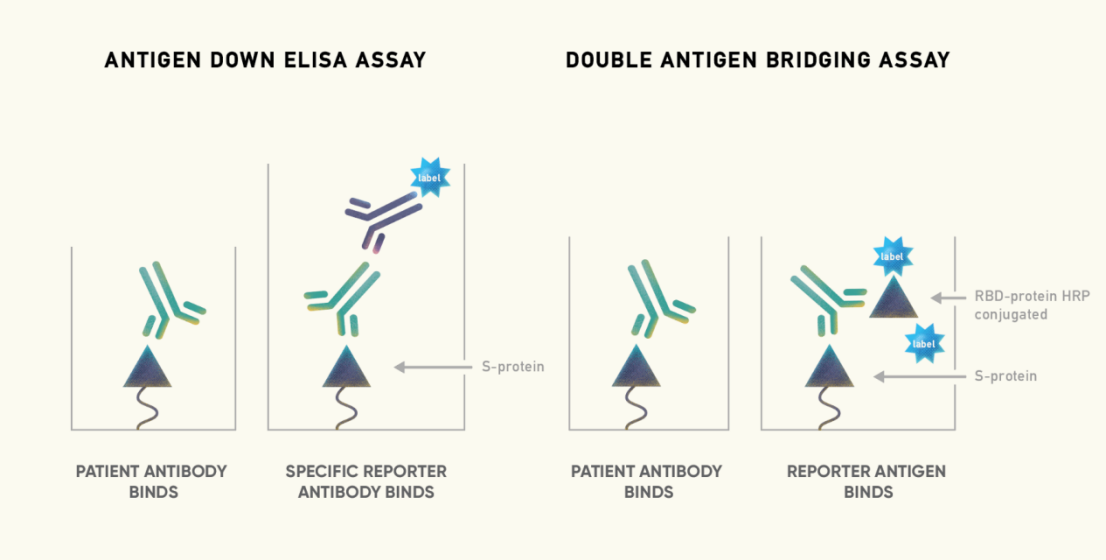


Figure 22. *Principles of the Antigen Down and Double Antigen Bridging Assay ELISA methods tested [**Meyer et al. 2017**].*

In the Antigen Down technique, the spike protein of SARS-CoV2-2019 (S) is immobilized on the ELISA plate and patient serum is applied. Immunoglobulins against S protein bind to the immobilized protein. Anti-human total Ig secondary antibody conjugated to horse radish peroxidase enzyme (HRP) binds to the immobilized patient's antibodies that may be present in the serum. Enzyme substrate is added (TMB), which in the presence of the HRP enzyme will be catalyzed forming a blue colored solution. The intensity of the color solution is proportional to the concentration of antibodies in the serum. The signal is measured at 450nm using a microplate photometer.

In contrast, in the Double Antigen ELISA, the immobilised S protein coating the ELISA plate is recognized by the patient's serum antibodies. According to recent bibliography, one of the most immunogenic portions of the S protein of the virus is the receptor binding domain (RBD) [**Zhao *et al.*, 2019; Du *et al.*, 2020**]. The RBD portion is responsible for ACE2 receptor binding of the virus to the host cells. In double antigen ELISA, RBD protein has been conjugated to HRP and added to the plate with the immobilised S protein. Antibodies in the serum of the patient that bind to the immobilised protein S will also bind to the RBD-HRP conjugated protein. Upon addition of TMB substrate, signal is measured as above.

5.2 Antigen production

Antibody detection against SARS-CoV-2 via ELISA requires antigen production through recombinant technologies and its immobilization onto a well-plate (**Figure 23**). In our case, an expression vector for protein expression in mammalian (eukaryotic) cells which encodes the S-viral protein was purchased from Lubio-Sino biologicals*. The latter was used to transform E. coli bacterial cells (DH5A strain) competent cells. The transformation process was undertaken through the heat-shock protocol as described in Sambrook and Russel (2001). Following transformation, bacteria are selected on agar plates containing kanamycin. Only bacteria carrying the plasmid are antibiotic-resistant and can form a colony. Colonies are then transferred to liquid cultures and the integrity and structure of the expression vector is confirmed through restriction enzyme digestion. Colonies that exhibit the correct fragment analysis are subsequently grown in order to generate large quantities of the purified plasmid, which will be used for the transfection of mammalian cells. Plasmid is isolated using NucleoBond Xtra Midi preparation kit (Macherey Nagel, Cat No: 740410.50). The purity and integrity of the final plasmid preparation is measured using a NanoDrop 2000c spectrophotometer (Thermo Scientific).

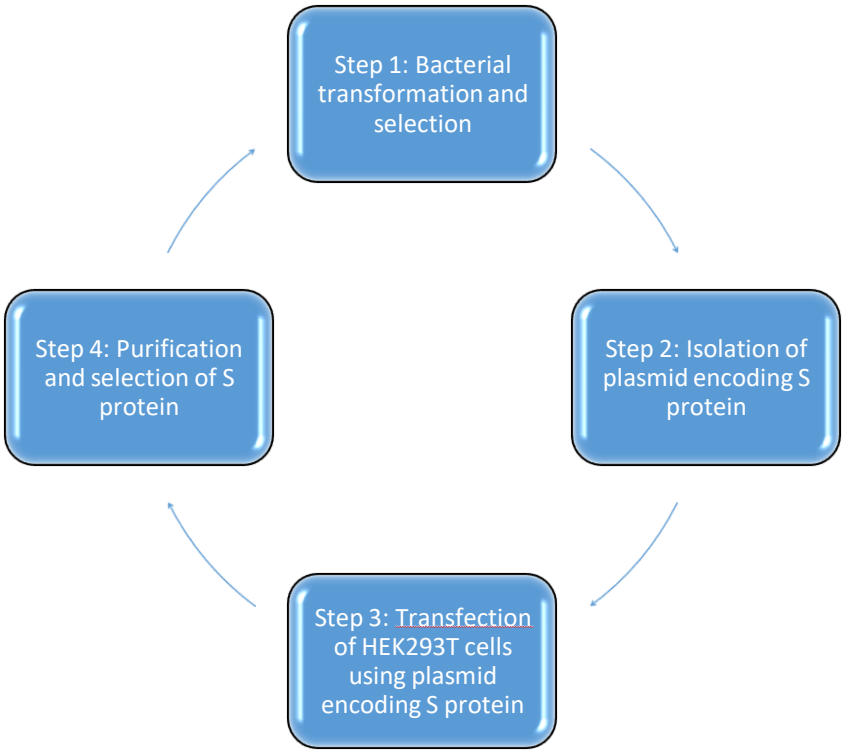


Figure 23. *Workflow diagram presenting antigen production process.*

Next step is the transfection of mammalian cell line HEK293T using the purified expression vector encoding the S-protein as per protocol. TransfeX Transfection Reagent was used according to manufacturer recommendations. Cells were passaged 48 hours post-transfection in complete growth medium containing Hygromycin B. Selection was maintained for 1 to 2 weeks to allow for selection of cells that have undergone stable integration. Transfected cells secrete the recombinant S protein in the supernatant which is isolated and purified using GE Healthcare TALON Superflow kit, according to manufacturer's instructions.

After protein elution, S protein is further purified according to its molecular weight using Amicon Ultra-4 Centrifugal Filters (MERCK Millipore, Cat.No. UFC8003). Following protein purification and collection, their concentration is measured using a NanoDrop spectrophotometer.

*__CAUTION__: Recent experiments in our lab showed that protein integrity using the Sino Biological SARS-CoV-2 2019 expression plasmids is not consistent. The final S and RBD proteins used in the test was provided by Trenzyme Gmbh.

5.3 Comparison of ELISA methods

In the Antigen Down ELISA assay, detection is based on an anti-human antibody conjugated to HRP whereas in the Double Antigen bridging assay, antigen RBD is conjugated to HRP (**Figure 22**). The former binds to the Fc while the latter to the Fab region of the anti-S human antibody, respectively. Thus, in Antigen Down, protein S is immobilized on the microplate well and patient serum is added. Subsequently, secondary antibody - conjugated to HRP that will bind to human antibodies - is added and presence is detected through solution color change.

5.4 Double Antigen ELISA protocol

The first step in the Double Antigen ELISA protocol is the immobilization of SARS-CoV-2 2019 S Protein in 96-well ELISA microplates; their flat bottom allows for high binding capacity. Polystyrene well-plates are used due to their enhanced intrinsic ability to form hydrogen bonds, which are necessary for protein attachment to the surface of the microplate well. Following protein immobilization, washing of the plate (x3) with PBS is necessary to remove the non-immobilized S protein. The next step includes a Bovine Serum Albumin (BSA) incubation period in order to saturate available protein-binding sites. After a washing cycle with PBS (x3), the excess amount of BSA is removed. Phosphate buffer incubation at 4^{o}C overnight follows in order to increase the affinity between S-viral protein and the well surface. Next, a washing cycle (x3) is performed with PBS to completely remove any residual buffer and then serum sample is loaded. Subsequently, additional washing (x5) with PBS-Tween (PBST) is done to remove any non-specific binding between the serum and the well surface. RBD-conjugated with HRP (a widely used photosensitive oxidizing enzyme) is then loaded and recognized by its cognate antibody. This is followed by an additional washing step (x5) with PBST to remove the unbound part of HRP-RBD complexes. Then, 3,3′,5,5′-Tetramethylbenzidine (TMB) peroxidase substrate is loaded and solution's color turns blue in case of a positive sample. In contrast, inability to detect any color change indicates a negative test. HRP and TMB are photosensitive meaning they are deactivated following exposure to light; thus, the above two steps following HRP addition take place in dark, while keeping light exposure to minimum. Termination of the reaction is achieved using phosphoric acid which changes the pH of the solution and thus inhibits the enzymatic activity of HRP. Following addition of phosphoric acid, the solution's color changes from blue to yellow (in case of a positive test) or remains colorless (in case of a negative test).

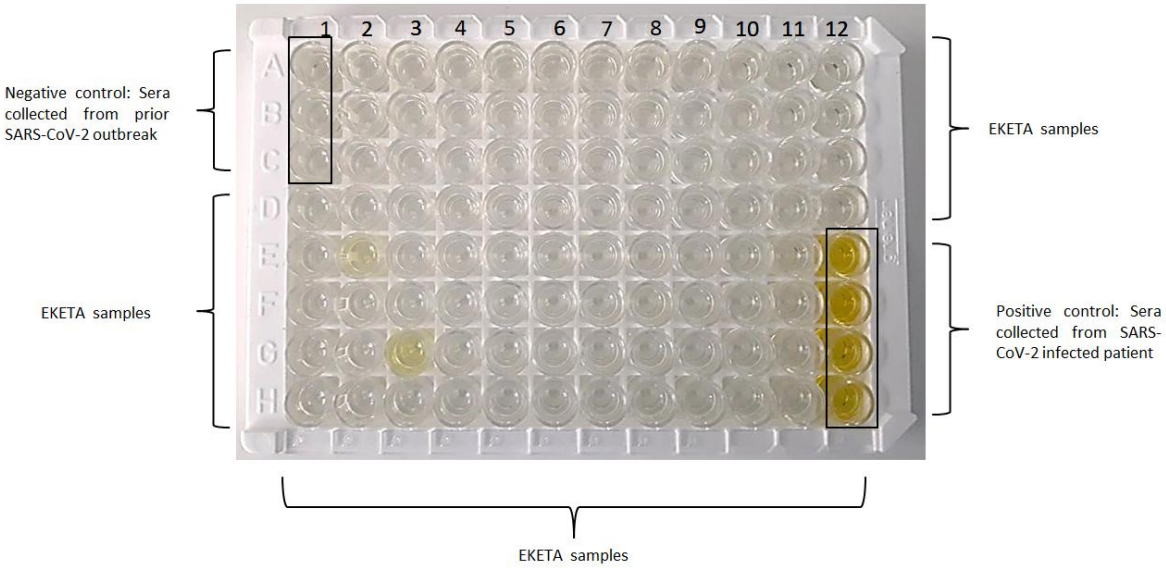
Finally, samples are loaded on a microplate photometer to determine the absorbance of each sample at a wavelength of 450 nm. Cut-off was set to 0.1.

The procedure followed is:

1. Coat plate with 100ul of 1,5ug/ml SARS-CoV-2 S protein in PBS and incubate O/N at 4^0 C
2. Wash plate 3 times with PBS
3. Add 300ul of 4% BSA in PBS and incubate at 37^0 C for 2 hours
4. Wash plate 3 times with 200ul PBS
5. Add 300ul of Phosphate buffer
6. Wash plate 3 times with 200ul PBS
7. Add 100ul of 1:1 of serum in PBS, incubate at RT for 1 hour
8. Wash with 200ul PBST 5 times
9. Add 100ul RBD:HRP (2:12.000) in PBST, incubate for 45min in dark at RT.
10. Wash 5 times with 200ul PBST
11. Add 100ul of TMB and incubate at RT for 15min
12. Add 100ul Phosphoric acid

5.5 Application of the double antigen ELISA protocol on clinical samples

152 clinical serum samples obtained from "EKETA" in Thessaloniki were tested. Results showed 3 to be positive and 149 to be negative. Representative image of the results is shown below (**Figure 24**). Samples tested positive for SARS-CoV-2 are indicated in Yellow. Following standardization, the cut-off value for a positive test was set to 0.1.

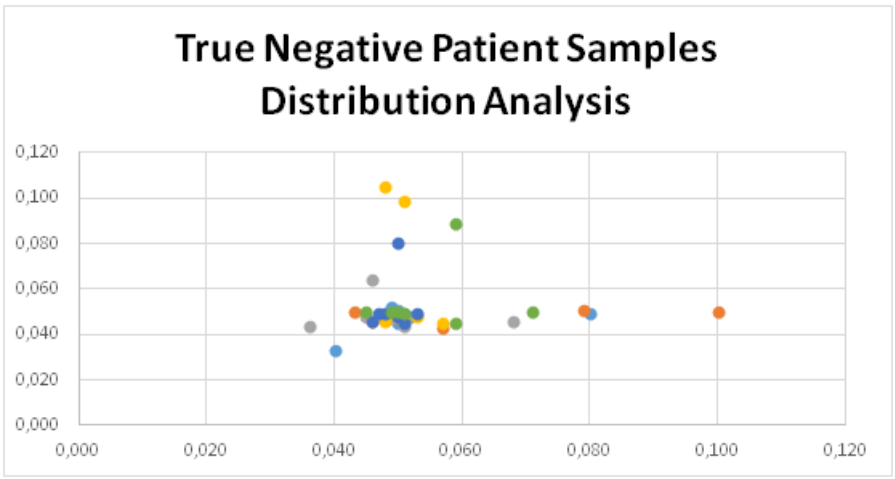


1st Batch	1	2	3	4	5	6	7	8	9	10	11	12
A	0,073	0,075	0,074	0,069	0,075	0,080	0,076	0,079	0,073	0,077	0,070	0,070
B	0,069	0,066	0,070	0,062	0,065	0,068	0,075	0,078	0,069	0,081	0,073	0,073
C	0,070	0,064	0,067	0,062	0,071	0,071	0,071	0,078	0,074	0,076	0,073	0,069
D	0,062	0,071	0,064	0,069	0,067	0,072	0,069	0,077	0,069	0,074	0,082	0,073
E	0,067	0,225	0,064	0,076	0,061	0,070	0,070	0,073	0,071	0,074	0,078	3,117
F	0,073	0,075	0,068	0,064	0,067	0,076	0,076	0,097	0,077	0,075	0,073	9,999
G	0,064	0,068	0,392	0,068	0,070	0,076	0,074	0,071	0,069	0,075	0,069	2,917
H	0,063	0,065	0,068	0,070	0,073	0,070	0,074	0,073	0,077	0,074	0,072	2,976

Figure 24. *Representative image of 96 well-plate following phosphoric acid incubation. (Cut off: 0.1) In the table below absorbance values for each well, are depicted (yellow highlighted values depict positive samples).*

5.6 Sensitivity and specificity of the double antigen ELISA protocol

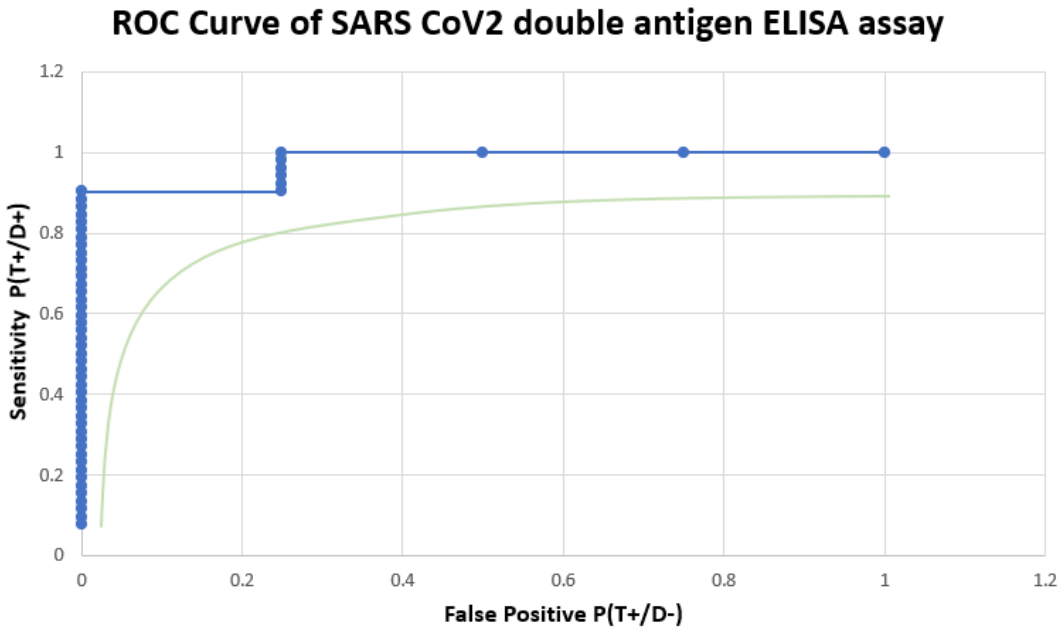
In order to determine the sensitivity and the specificity of the ELISA assay, a ROC analysis was performed. To ensure that samples used in our analysis are all true negatives, we used sera patient samples collected before the SARS-CoV-2 2019 pandemic (**Figure 25**). We followed the double antigen protocol as described above.



Mean: 0,053; SD: 0,013; Cut-Off: 0,092

1st Batch	1	2	3	4	5	6	7	8	9	10	11	12
A	**0,080**	**0,049**	**0,050**	**0,047**	**0,049**	**0,047**	**0,048**	**0,046**	**0,046**	**0,046**	**0,050**	**0,050**
B	**0,049**	**0,052**	**0,048**	**0,047**	**0,050**	**0,050**	**0,051**	**0,046**	**0,050**	**0,080**	**0,059**	**0,045**
C	**0,051**	**0,049**	**0,046**	**0,046**	**0,051**	**0,044**	**0,053**	**0,048**	**0,047**	**0,049**	**0,045**	**0,050**
D	**0,050**	**0,045**	**0,079**	**0,051**	**0,046**	**0,064**	**0,048**	**0,105**	**0,051**	**0,045**	**0,071**	**0,050**
E	**0,050**	**0,051**	**0,100**	**0,050**	**0,045**	**0,048**	**0,051**	**0,099**	**0,053**	**0,049**	**0,059**	**0,089**
F	**0,052**	**0,048**	**0,043**	**0,050**	**0,068**	**0,046**	**0,048**	**0,048**	**0,050**	**0,048**	**0,049**	**0,050**
G	**0,040**	**0,033**	**0,057**	**0,043**	**0,036**	**0,044**	**0,057**	**0,045**	**0,048**	**0,049**	**0,051**	**0,049**
H	**0,080**	**0,049**	**0,050**	**0,047**	**0,049**	**0,047**	**0,048**	**0,046**	**0,046**	**0,046**	**0,050**	**0,050**

Figure 25. *ELISA double antigen assay of 96 true negative samples*



Sensitivity: 90,5; Specificity: 91,3; Criterion: 0,092

Figure 26. *ROC curve analysis of the sensitivity and the specificity of the double antigen ELISA assay for SARS CoV2 2019.*

The present ROC analysis determined that the sensitivity and specificity of the test is more than 90%, with a cut-off value of 0,092 (**Figure 26**).

6. Concluding Remarks

The evolving pandemic of the new coronavirus SARS-CoV-2 represents a major, public health issue. Hence, the scientific community has taken action to prevent from a future recurrence of this situation. The development of *"in house"* accurate and reliable molecular and immunological methods is already in progress within the frame of the "Emblematic action to handle SARS-CoV-2 infection: Epidemiological study in Greece via extensive testing for viral and antibody detection, sequencing of the virome and genetic analysis of the carriers".

Similar to other actions undertaken in different countries, this scientific approach aims to ensure diagnostic sufficiency in our country through the detection of possible new cases, monitoring their contacts. Molecular diagnostic tests focus on the identification of the viral RNA, while the immunological methods will allow the detection of antibodies against the virus in patient's blood serum as well as the development of monoclonal antibodies to identify the virus in biological samples.

Mapping of the mutations of the viral genome, and genomic analysis of the host are anticipated to shed light on genetic features, putatively associated with the high transmissibility and prevalence of infection, clinical severity and response to therapies. The Partners of this Consortium aim to contribute to the SARS-CoV-2 research in order to facilitate handling of the SARS-CoV-2 epidemic outbreak in Greece, by generating a specialized biobank and database comprising asymptomatic disease carriers and COVID-19 patients. The geographical contribution of the positive cases and the time-related tracking of the virus, allow the State to make decisions and to proceed with appropriate public health interventions.

Through this handbook, the highlights of this exhausting scientific effort held in the last trimester, are being presented. The results of this study are promising and their impact is expected to be significant for clinical and research purposes, in the near future.

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8. Appendixes

Appendix 1: Solutions and reagents for AGPC-based method for RNA extraction

a. Protocols for solutions preparation:

DEPC treated water

1. Treat double distilled water with 0.1% vol /vol DEPC by vigorous stirring for at least 2 hours at 37 °C in fume hood.

    Example: add 1 ml DEPC to 1 lt of double distilled water and stir on a heater.

2. Autoclave, (at least 15 min) to inactivate traces of DEPC.

    Suggestion: perform the whole procedure in a dark, large volume, i.e. 2 l glass bottle, from start up to autoclave step.

Denaturing solution (solution D)

Denaturing solution (solution D) is 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% (wt/vol) N-laurosylsarcosine (Sarkosyl) and 0.1 M 2-mercaptoethanol.

1) You can prepare a stock solution by dissolving 250 g guanidinium thiocyanate in 293 ml water at 65 °C.

2) Then you add 17.6 ml of 0.75 M sodium citrate, pH 7.0, and 26.4 ml of 10% (wt/vol) Sarkosyl. The stock solution can be stored up to 3 months at 25 °C (room temperature).

3) To prepare the working solution D, just add 0.36 ml of 98% 2-mercaptoethanol to 50 ml of stock solution.

Working solution D can be stored up to 1 month at room temperature.

! CAUTION: To minimize handling of guanidinium thiocyanate, dissolve directly in the manufacturer's bottle. 2-mercaptoethanol should be handled under a fume hood.

2 M sodium acetate, pH 4.0

1) Add 16.42 g sodium acetate (anhydrous) to 40 ml water and 35 ml glacial acetic acid.

2) Adjust to a pH of 4.0 with glacial acetic acid and bring to a final volume of 100 ml with DEPC-treated water.

The solution will be 2M with respect to sodium ions. Store up to 1 year at room temperature.

! CAUTION: Glacial acetic acid should be handled in a fume hood.

Water-saturated phenol

Dissolve 100 g phenol crystals (nucleic acid grade) in RNAse-free distilled water at 65 °C. Specifically, start heating the bottle containing the phenol crystals in a water bath at 65 °C and gradually add 100 ml of RNase-free water. When phenol has melted, you can add a magnet and initiate mild stirring. After melting has been completed, let cool, aspirate most of the upper water phase and store up to 1 month at 4 °C. For larger amounts of crystal phenol (i.e. 500 mg) you may split into 100 ml aliquots at the end of the procedure.

! CAUTION: Phenol should be handled under a fume hood.

CRITICAL STEP: The acidic pH is the critical factor to ensure the separation of RNA from DNA and proteins. For this reason, never use buffered phenol instead of water-saturated phenol, and measure the pH of the upper water phase with pH-indicator strips (avoid using pH meter as phenol may damage the electrode).

Chloroform:isoamyl alcohol (49:1, vol/vol)

Mix 49 ml of chloroform with 1 ml of isoamyl alcohol.

CRITICAL: Prepare just before use.

! CAUTION: This should be handled under a fume hood.

Note: Chloroform:isoamyl alcohol (49:1, vol/vol) is already available under order code 25668 Sigma-Aldrich / Merck

Isopropanol
Use straight from the manufacturer's bottle.

75% ethanol
Add 75 ml absolute ethanol to 25 ml DEPC-treated water.

0.5% sodium dodecyl sulfate (SDS)
Dilute 1 ml of 10% SDS in 19 ml of DEPC treated water. Store at room temperature.
! CAUTION: A mask should be worn while weighing SDS.

tRNA solution preparation (in case dry tRNA is used)
Prepare a solution containing yeast tRNA at a concentration of 10 mg/ml in sterile TE (pH 7.6), 0.1 M NaCl. Extract the solution twice with phenol (equilibrated in Tris-Cl at pH 7.6) and twice with chloroform. Precipitate the RNA with 2.5 volumes of ethanol at room temperature, and recover the RNA by centrifugation at 5000g for 15 minutes at 4°C. Dissolve the pellet of RNA at a concentration of 10 mg/ml in sterile TE (pH 7.6). Store the carrier RNA in small aliquots at -20°C.

Other important procedures
CRITICAL: Take maximum care not to contaminate your samples with RNases.

1) For this reason you must use pipettes (including automatic ones) reserved for RNA work to prevent cross-contamination with RNases from other activties.

2) Always wear disposable gloves, as cells from the skin as well as bacteria and molds can contaminate your samples and can be sources of RNases.

3) Use either disposable, sterile plasticware or non-disposable glassware or plasticware that must be RNase-free. For this, glassware can be baked at 150°C for 4 h and plasticware can be soaked for 10 min in 0.5 M NaOH, rinsed with water and autoclaved.

Reference: Sambrook and Russel, 2001.

b. Reagents – Consumables for solutions preparation:

- DEPC (Diethyl pyrocarbonate): D5758 Sigma-Aldrich / Merck
  ! CAUTION: Harmful, wear lab coat, gloves and perform treatments in fume hood.
  (ready to use UltraPure™ DEPC-Treated Water: 750023 ThermoFisher Scientific)

- Guanidinium thiocyanate: 1.04167 Millipore/Merck; 8.20613 Sigma-Aldrich / Merck; AM9422 ThermoFisher Scientific
  ! CAUTION: Harmful, wear lab coat, gloves and perform treatments in fume hood.

- Sodium citrate: W302600 Sigma-Aldrich / Merck

- N-laurosylsarcosine (Sarkosyl): L5000 Sigma-Aldrich / Merck
  ! CAUTION: wear mask to avoid inhaling.

- 2-mercaptoethanol: 21985023 ThermoFisher Scientific; M3148 Sigma-Aldrich / Merck

- Sodium acetate (anhydrous): S2889 Sigma-Aldrich / Merck

- Glacial acetic acid: 33209-M Sigma-Aldrich / Merck

- Phenol (nucleic acid grade): 15509037 ThermoFisher Scientific
  ! CAUTION: Do not inhale, corrosive. Wear lab coat, gloves and perform treatments in fume hood.

- Chloroform: C2432 Sigma-Aldrich / Merck
  Note: Chloroform:isoamyl alcohol (49:1, vol/vol) is already available under order code 25668 Sigma-Aldrich / Merck

- Isoamyl alcohol: W205702 Sigma-Aldrich / Merck

- Isopropanol (2-Propanol): I9516 Sigma-Aldrich / Merck

- Ethanol: 493511 Sigma-Aldrich / Merck

- Sodium dodecyl sulfate (SDS): L3771, 71725 Sigma-Aldrich / Merck
  ! CAUTION: wear mask to avoid inhaling.

- NaOH: S8045 Sigma-Aldrich / Merck
  ! CAUTION: corrosive.

- (yeast)tRNA: 15401029 (50 mg), 15401011 (25 mg), AM7119 (500 µl: 10 mg/mL) ThermoFisher Scientific; 10109495001 (100 mg), 10109509001 (500 mg) Sigma-Aldrich / Merck

- Glycogen: R0551 ThermoFisher Scientific

- Trizol: 15596018 ThermoFisher Scientific

- Trizol LS: 10296028 ThermoFisher Scientific

- pH-indicator strips: 1.09584 (pH 2.0 - 9.0), 1.09531 (pH 0 - 6.0) Sigma-Aldrich / Merck

Appendix 2: Materials and reagents for the "Double Antigen" ELISA protocol

a. Reagents and kit for antigen production and "Double Antigen" ELISA protocol

- DH5α bacteria
- 293T cell line 12022001 Sigma-Aldrich / Merck
- NucleoBond Xtra Midi kit for transfection-grade plasmid DNA Macherey-Nagel
- Amicon® Ultra-4 Centrifugal Filter Units Merckmillipore
- NanoDrop spectrophotometer
- TransfeX™ Transfection Reagent: ACS-4005™ ATCC
- Hygromycin B 31282-04-9 Sigma-Aldrich / Merck
- His GraviTrap™ TALON® GE29-0005-94 Sigma-Aldrich / Merck
- Isopropanol (2-Propanol): I9516 Sigma-Aldrich / Merck
- Ethanol: 493511 Sigma-Aldrich / Merck
- Greiner High Binding plates Microlon 96 well M4561 Sigma-Aldrich / Merck
- Novus Biologicals™ Lightning-Link™ HRP Antibody Labeling Kit 7010004 ThermoFisher Scientific
- Gibco™ PBS Tablets 18912014 ThermoFisher Scientific
- Na_2HPO_4 10049-21-5 Sigma-Aldrich / Merck
- NaCl S9888 Sigma-Aldrich / Merck
- BSA 9048-46-8 Sigma-Aldrich / Merck
- Wash Buffer 20X PROGNOSIS BIOTECH
- 3,3′,5,5′-Tetramethylbenzidine (TMB)
- Phosphoric acid 7664-38-2 Sigma-Aldrich / Merck

b. Protocols for solutions preparation:

PBS

- Dissolve one PBS tablet in 500 ml of distilled water

Wash buffer

- Dilute Wash buffer 20X to 1X using distilled water

Phosphate buffer

- Prepare a mix of 50mM $NaHPO_4$, 100mM NaCl and 1% BSA

Phosphoric acid

- Dilute Phosphoric acid 100 % stock at final concentration of 17%

HRP conjugated S-protein

- Dilute purified S protein (from antigen production process) in wash buffer (PBST) at final concentration of 2:12,000 ml in dark conditions)