

DETECTING A VIRUS

The invention provides a method for detection of a coronavirus. In particular, the invention provides a method for detecting the novel 2019-nCoV coronavirus (now known as
5 COVID-19 virus, or SARS-CoV-2) utilising the reverse transcription polymerase chain reaction (RT-PCR) technique. As such, the invention has been developed using specific primers and/or probes to effectively detect a coronavirus.

Currently, there is a great need for the development of new detection method that are
10 effective in diagnosing viral infections, particularly against viral infections which are associated with high morbidity and mortality, and which impact on sizable populations. Testing methods currently available are inadequate or ineffective in large proportions of infected patients.

15 A further well-known family of pathogenic viruses are the Coronaviruses. Coronaviruses (Order Nidovirales, family Coronaviridae, Genus Coronavirus) are enveloped positive-stranded RNA viruses that bud from the endoplasmic reticulum-Golgi intermediate compartment or the cis-Golgi network (Fischer, Stegen et al. 1998; Maeda, Maeda et al. 1999; Corse and Machamer 2000; Maeda, Repass et al. 2001; Kuo and Masters 2003).

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Coronaviruses infect humans and animals and it is thought that there could be a coronavirus that infects every animal. The two human coronaviruses, 229E and OC43, are known to be the major causes of the common cold and can occasionally cause pneumonia in older adults, neonates, or immunocompromised patients (Peiris, Lai et al.
25 2003). Animal coronaviruses can cause respiratory, gastrointestinal, neurological, or hepatic diseases in their host (Peiris, Lai et al. 2003). Several animal coronavirus are significant veterinary pathogens (Rota, Oberste et al. 2003).

Severe acute respiratory syndrome (SARS) is caused by a newly identified virus. SARS is
30 a respiratory illness that has recently been reported in Asia, North America, and Europe (Peiris, Lai et al. 2003). The causative agent of SARS was identified as a coronavirus (SARS-CoV-1). (Drosten, Gunther et al. 2003; Ksiazek, Erdman et al. 2003; Peiris, Lai et al. 2003). The World Health Organization reports that the cumulative number of reported

probable cases of SARS from 1 November 2002 to the 11th July 2003 is 8,437 with 813 deaths, nearly a 10% death rate. It is believed that SARS will not be eradicated, but will cause seasonal epidemics like the cold or influenza viruses (Vogel 2003).

- 5 Now, a novel coronavirus (nCoV) is a new coronavirus that has not been previously identified. This 2019 novel coronavirus (2019-nCoV or SARS-CoV-2), initially known as BetaCoronavirus/Wuhan, is not the same as the coronaviruses that commonly circulate among humans and cause mild illness, like the common cold.
- 10 There is a need to develop a method for the quick identification and detection of the virus in patients who may be infected or exposed to the virus in order to prevent the virus from spreading in the community. However, a unique target region of the nCoV needs to be identified. The target region should be distinguishable from other related coronaviruses and be less prone or resistant to mutations to allow the amplification and detection of the
- 15 target region to be able to effectively detect the presence of the nCoV and possible future mutations.

The listing or discussion of an apparently prior-published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the

20 state of the art or is common general knowledge.

Any document referred to herein is hereby incorporated by reference in its entirety.

- In an aspect of the invention, there is provided compositions and methods for detecting the
- 25 presence of the coronavirus 2019-nCoV in a biological sample. In particular, said detection includes detecting an RNA of the 2019-nCoV virus in said biological sample. The 2019-nCoV viral RNA may also be referred to as "target RNA" in this application.

- As such, this invention is a highly sensitive and specific Taqman-based RT-PCR assay for
- 30 the detection of COVID-19 virus. This assay may be used as a single-plex or multiplex assay together with other targets such as Influenza A, Influenza B, 2009-H1N1 subtype or H3N2 subtype etc. The assay of the present invention is also made for the detection of SARS-CoV-2, also called BetaCoV/Wuhan.

TaqMan-based real-time polymerase chain reaction (real-time PCR) is employed, which uses a sequence-specific DNA probe consisting of oligonucleotides labelled with a fluorescent reporter, to monitor the amplification of a targeted DNA molecule during the PCR (i.e., in real time).

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In various embodiments, the detection method is carried out using an RT-PCR technique.

Reverse transcriptases have been extensively used in reverse transcribing RNA prior to PCR amplification. This method, often referred to as RNA-PCR or RT-PCR, is used for
10 detection and quantitation of RNA.

A number of protocols have been developed taking into account the three basic steps of the procedure: (a) the denaturation of RNA and the hybridization of reverse primer; (b) the synthesis of cDNA; and (c) PCR amplification. In the so-called "uncoupled" RT-PCR procedure (e.g., two-step RT-PCR), reverse transcription is performed as an
15 independent step using the optimal buffer condition for reverse transcriptase activity. Following cDNA synthesis, the reaction is diluted to decrease $MgCl_2$ and deoxyribonucleoside triphosphate (dNTP) concentrations to conditions optimal for Taq DNA Polymerase activity, and PCR is carried out according to standard conditions (see
20 U.S. Patent Nos. 4,683,195 and 4,683,202). By contrast, "coupled" RT-PCR methods use a common or compromised buffer for reverse transcriptase and Taq DNA Polymerase activities. In one version, the annealing of reverse primer is a separate step preceding the addition of enzymes, which are then added to the single reaction vessel. In another version, the reverse transcriptase activity is a component of the thermostable Tth DNA polymerase.
25 Annealing and cDNA synthesis are performed in the presence of Mn^{++} , then PCR is carried out in the presence of Mg^{++} after the removal of Mn^{++} by a chelating agent. Finally, the "continuous" method (e.g., one-step RT-PCR) integrates the three RT-PCR steps into a single continuous reaction that avoids the opening of the reaction tube for component or enzyme addition. Continuous RT-PCR has been described as a single
30 enzyme system using the reverse transcriptase activity of thermostable Taq DNA Polymerase and Tth polymerase and as a two-enzyme system using AMV-RT and Taq DNA Polymerase wherein the initial 65°C RNA denaturation step was omitted.

The RT-PCR of the methods described herein may be a one-step or two-step process.

In various embodiments, the primers utilised in the RT-PCR method (particularly in a 2-plex detection kit) are set out below:

5 **Target set: nCoV(113)**

nCoV(113)F:(5'-3')TGTATACGTTGTCTTTGGAGCACA (SEQ ID No. 1), Standard desalting

nCoV(113)R:(5'-3')GGTTTTAGATCTTCGCAGGCAAG (SEQ ID No. 2), Standard desalting

10 nCoV(113)LO:(6FAM)5'-CCATTCCCTGCGCGTCCTC-3'(BHQ1), HPLC grade
CCATTCCCTGCGCGTCCTC (SEQ ID No. 3)

nCoV(118)F: (5'-3')CCTGGTGTATACGTTGTCTTTGGAG, (SEQ ID No. 9), Standard desalting (IDT)

15 **IC set:**

IC77F(22):(5'-3')GCGCCCGAACAGTATCGCGTTT (SEQ ID No. 4), Standard desalting

IC77R(20):(5'-3')AGCCTCCGCTAGTCACCCAA (SEQ ID No. 5), Standard desalting

IC77P(22):(5'-3') (HEX)5'-AGGTCGGGTGGGCGGGTCGTTA-3'(BHQ1), HPLC grade
AGGTCGGGTGGGCGGGTCGTTA (SEQ ID No. 6)

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IC Template sequence:

(5'-3')

CTAGCAGTGGCGCCCGAACAGTATCGCGTTTATGCGAGGTCGGGTGGGCGGGT
CGTTAGTTTCGTTTTGGGTGACTAGCGGAGGCTAGAAGGAGAGAGATGG

25 Len=102, (SEQ ID No. 7)

Positive control Template sequence:

(5'-3')

TGTATACGTTGTCTTTGGAGCACAAAACCAGTTGAAACATCAAATTCGTTTGAT
30 GTACTGAAGTCAGAGGACGCGCAGGGAATGGATAATCTTGCCTGCGAAGATCT
AAAACC

Len=113, (SEQ ID No. 8)

In an embodiment, there is provided a PCR primer set for a target region, the PCR primer set comprising a forward primer selected from the group consisting of SEQ ID No. 1, SEQ ID No. 9, SEQ ID No. 10, and SEQ ID No. 11, and a reverse primer of SEQ ID No. 2 when one of SEQ ID No. 1, SEQ ID No. 9, or SEQ ID No. 10 is selected as the forward primer or SEQ ID No. 12 when SEQ ID No. 11 is selected as the forward primer. The primers may be synthesised by any known method and may be modified without losing its activity. More preferably, the primers consist essentially of the sequences above. Even more preferably, the primers consist of the sequences above.

5 Preferably, the forward primer is SEQ ID No. 1 and the reverse primer is SEQ ID No. 2.

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Preferably, both SEQ ID No. 1 and SEQ ID No. 9 are selected as the forward primers.

The PCR primer set may further comprise a TaqMan probe. The TaqMan probe is generally made up of a fluorophore and a quencher flanking a sequence which anneals to part of the target region. The TaqMan probe helps in the quantitative PCR detection and analysis. An example of a sequence that may be used is SEQ ID No. 3 when one of SEQ ID No. 1, SEQ ID No. 9, or SEQ ID No. 10 is selected as the forward primer or SEQ ID No. 13 when SEQ ID No. 11 is selected as the forward primer.

15 20 Other fluorophores and quenchers may be used as appropriate depending on the type of PCR and potentially other probes present. Non-limiting examples of fluorophores that may be used include 6-carboxyfluorescein (6-FAM), tetrachlorofluorescein (TET), 6-Carboxy-4',5'-Dichloro-2',7'-Dimethoxyfluorescein (JOE), hexachlorofluorescein (HEX), carboxyrhodamine (ROX), Cy3, and Cy5. Non-limiting examples of quenchers that may be used include black hole quenchers 1, 2 and 3 (BHQ-1, BHQ-2, BHQ-3), N-[4-(4-dimethylamino)phenylazo]benzoic acid (DABCYL), and carboxytetramethylrhodamine (TAMRA). Suitability of the fluorophore and quencher depends on whether a single or multiplex PCR is to be used as the different fluorophores have different excitation and emission wavelengths while the different quenchers quench at different wavelengths and may have passive emissions which needs to be taken into consideration.

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In another aspect, there is provided a method of detecting the presence of a coronavirus in a sample, the method comprises: providing a PCR primer set, the PCR primer set comprising a forward primer selected from the group consisting of SEQ ID No. 1, SEQ ID

No. 9, SEQ ID No. 10, and SEQ ID No. 11, and a reverse primer of SEQ ID No. 2 when one of SEQ ID No. 1, SEQ ID No. 9, or SEQ ID No. 10 is selected as the forward primer or SEQ ID No. 12 when SEQ ID No. 11 is selected as the forward primer; testing a sample with the PCR primer set using a reverse transcription polymerase chain reaction to
5 amplify a target region of the coronavirus DNA present in the sample; and measuring a level of the target region to determine if the coronavirus DNA is present in the sample. The PCR primer set used in the method may further contain the other preferred embodiments and examples of the PCR primer set described above.

10 In another aspect, there is provided a kit comprising a forward primer selected from the group consisting of SEQ ID No. 1, SEQ ID No. 9, SEQ ID No. 10, and SEQ ID No. 11, and a reverse primer of SEQ ID No. 2 when one of SEQ ID No. 1, SEQ ID No. 9, or SEQ ID No. 10 is selected as the forward primer or SEQ ID No. 12 when SEQ ID No. 11 is selected as the forward primer, wherein the forward primer and the reverse primer bound a
15 target region; a first TaqMan probe that anneals to part of the target region; a set of internal control primers to target an internal control template sequence, and a second TaqMan probe that anneals to part of the internal control template sequence.

Preferably the forward primer is SEQ ID No. 1 and the reverse primer is SEQ ID No. 2.

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Preferably both SEQ ID No. 1 and SEQ ID No. 9 are selected as the forward primers.

Preferably the TaqMan probe comprises SEQ ID No. 3 when one of SEQ ID No. 1, SEQ ID No. 9, or SEQ ID No. 10 is selected as the forward primer or SEQ ID No. 13 when
25 SEQ ID No. 11 is selected as the forward primer, a fluorophore and a quencher. Examples of the fluorophore and quencher that may be used are as with the PCR primer set.

Preferably the primers and TaqMan probes are in solution with an identical concentration.

30 Preferably the kit further comprises at least one (more preferably the kit comprises most, and even more preferably the kit comprises all) of the following: Taq DNA polymerase, deoxyribonucleotides, magnesium chloride, the internal control template sequence, and nuclease free water.

Advantageously, using the primers described herein, in particular with the TaqMan probe, a single tube RT-PCT assay may be performed with a PCR cycling time of about 1 hour 40 minutes. This is faster and uses less reagents compared to existing methods, such as the pan-coronavirus PCR followed by sequencing to identify the virus and assays using two
5 tubes for each sample which consumes substantially more reagents.

By "Polymerase Chain Reaction (PCR)", it is meant to refer to the technique of repeatedly heating and cooling a sample solution containing nucleic acids to successively replicate a portion having a specific base sequence of a nucleic acid and exponentially amplifying the
10 nucleic acid having the portion of the specific base sequence, and specifically, it may be progressed to a series of temperature enzyme reaction steps including denaturation, annealing, extension and the like. Two- or three-step thermal cycling (e.g., 90-96 °C, 37-55 °C and 72 °C) may be used for the reaction steps described. PCR is widely used in life science, genetic engineering, medical field and the like for analysis and diagnosis
15 purposes.

The terms "polynucleotide", "oligonucleotide", "nucleic acid" and "nucleic acid molecule" are used interchangeably herein to refer to a polymeric form of nucleotides of any length, and may comprise ribonucleotides, deoxyribonucleotides, analogues thereof, or mixtures
20 thereof. Whether modified or unmodified, in some embodiments the target nucleotide must have a polyanionic backbone, preferably a sugar-phosphate backbone. More particularly, the terms "polynucleotide", "oligonucleotide", "nucleic acid" and "nucleic acid molecule" include polydeoxyribonucleotides (containing 2-deoxy-D-ribose), polyribonucleotides (containing D-ribose), including tRNA, rRNA, hRNA, and mRNA, whether spliced or
25 unspliced, any other type of polynucleotide which is an N or C-glycoside of a purine or pyrimidine base, and other polymers containing a phosphate or other polyanionic backbone, and other synthetic sequence-specific nucleic acid polymers providing that the polymers contain nucleobases in a configuration which allows for base pairing and base stacking, such as is found in DNA and RNA. These terms include, for example, 3'-deoxy-
30 2',5'-DNA, oligodeoxyribonucleotide N3'→P5' phosphoramidates, 2'-O-alkyl-substituted RNA, double- and single-stranded DNA, as well as double- and single-stranded RNA, and hybrids thereof including for example hybrids between DNA and RNA, and also include known types of modifications, for example, labels, alkylation, caps. Substitution of one or more of the nucleotides with an analog, internucleotide modifications such as, for example,

those with negatively charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (including enzymes (e.g. nucleases), toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelates (of, e.g., metals, radioactive
5 metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide or oligonucleotide.

The terms "nucleoside" and "nucleotide" will include those moieties which contain not only
10 the known purine and pyrimidine bases, but also other heterocyclic bases which have been modified. Such modifications include methylated purines or pyrimidines, acylated purines or pyrimidines, or other heterocycles. Modified nucleosides or nucleotides can also include modifications on the Sugar moiety, e.g., wherein one or more of the hydroxyl groups are replaced with halogen, aliphatic groups, or are functionalized as ethers, amines, or the like.
15 The term "nucleotidic unit" is intended to encompass nucleosides and nucleotides.

In an embodiment, there is provided a kit comprising a forward primer selected from the group consisting of SEQ ID No. 1 and SEQ ID No. 9; a reverse primer of SEQ ID No. 2, wherein the forward primer and the reverse primer bound a target region; a first TaqMan
20 probe that anneals to part of the target region; a set of internal control primers to target an internal control template sequence, and a second TaqMan probe that anneals to part of the internal control template sequence. Preferably, the primers and TaqMan probes are in solution with an identical concentration. The kit may further comprise at least one, or more preferably most or all, of the following: Taq DNA polymerase, deoxyribonucleotides,
25 magnesium chloride, the internal control template sequence, and nuclease free water.

In an embodiment, there is provided a method of detecting the presence of coronavirus in a sample using the PCR primer set or kit described above. The method comprises: providing a PCR primer set, the PCR primer set comprising a forward primer selected
30 from the group consisting of SEQ ID No. 1 and SEQ ID No. 9, and a reverse primer of SEQ ID No. 2; testing a sample with the PCR primer set using a reverse transcription polymerase chain reaction to amplify a target region of coronavirus DNA present in the sample; and measuring a level of the target region to determine if the coronavirus DNA is present in the sample.

Preferably, both SEQ ID No. 1 and SEQ ID No. 9 are selected as the forward primers. Preferably, the PCR primer set further comprises a TaqMan probe.

- 5 Accordingly, a technique of simultaneously analysing nucleic acids of many templates from the same sample is needed, and such an analysis technique may be referred to as multiplex PCR. The RT-PCR of the invention may be carried out using multiple primers or, what may be known as a “multiplex polymerase chain reaction” (multiplex PCR). This refers to the use of polymerase chain reaction to amplify several different DNA sequences
- 10 simultaneously (as if performing many separate PCR reactions all together in one reaction). This process amplifies DNA in samples using multiple primers and a temperature-mediated DNA polymerase in a thermal cycler. The primer design for all primers pairs has to be optimized so that all primer pairs can work at the same annealing temperature during PCR. Also, the PCR of the invention may be carried out in real time. A
- 15 real-time polymerase chain reaction, also known as quantitative polymerase chain reaction, is a laboratory technique of molecular biology based on the polymerase chain reaction. It monitors the amplification of a targeted DNA molecule during the PCR, not at its end, as in conventional PCR.
- 20 By “detecting the presence”, it is meant to also include the measurement of the virus level, including expression levels of the RNA, in said biological sample. The presence and/or quantity of RNA is detected or measured, as the case may be, using primers or probes disclosed in this application. Examples of the assay methods available for detecting and measuring the RNA level include RT-PCR, competitive RT-PCR, real-time RT-PCR,
- 25 RNase protection assay (RPA), and Northern blotting, or a method using a DNA microarray chip, but are not limited thereto. The RNA level in a normal control group may be compared with that of the biological sample obtained from a subject.

- The detection and measurement of 2019-nCoV may be achieved preferably using RT-
- 30 PCR with primers specific to a portion of the virus genome. RT-PCR is a method that has been introduced by P. Seeburg (Cold Spring Harb Symp Quant Biol 1986, Pt 1:669-677) to analyze RNA, and in this regard, cDNA obtained by reverse transcription of mRNA is amplified by PCR for analysis. Here, in the amplification step, pairs of primers designed to be specific to the 2019-nCoV RNA are used. After RT-PCR, expression of 2019-nCoV

RNA and expression level thereof may be determined by examining the patterns and thicknesses of the bands separated upon electrophoresis. In addition, the mRNA expression level of the IGFBP5 gene may be compared with that of the normal control group. More importantly, after RT-PCR, the presence of 2019-nCoV RNA can be detected.

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The term "primer" used herein refers to a short strand of nucleic acid sequence which can form base pairings with a complementary template and has a free 3'-hydroxyl group serving as a starting point for replication of template strands. DNA synthesis can start with primers in the presence of a polymerase reagent (e.g., DNA polymerase or reverse transcriptase) under the proper conditions of buffer solutions and temperatures, and 4 different nucleoside triphosphates.

In an embodiment of the present invention, a 2019-nCoV RNA target gene may be amplified by PCR using a set of sense and antisense primers that are specific to the target gene and may have 7 to 50 nucleotide sequences. Here, the set of sense and antisense primers may further include additional features so long as the basic properties of the primers acting as a starting point of DNA synthesis are not changed. PCR conditions and lengths of sense and antisense primers may be appropriately selected according to techniques known in the art.

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The term "probe" used herein refers to a nucleic acid fragment, such as a DNA or RNA fragment, formed of from a few bases to several hundred bases. The probe may be labelled to determine the presence or absence of a target gene (RNA) and quantity (expression level) of the target gene. The probe may be manufactured in the form of an oligonucleotide probe, a single-stranded DNA probe, a double-stranded DNA probe, or an RNA probe.

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The primers or probes of the invention may be chemically synthesized using a phosphoramidite solid-phase method or another well-known method. In addition, these nucleic acid sequences (i.e., primers or probes) may be modified using methods known in the art. Examples of the modification are methylation, capping, substitution with at least one analogue of natural nucleotides, and internucleosidic modification, for example, modification with non-charged linkers (e.g., methylphosphonate, phosphotriester, phosphoroamidate, carbamate, etc) or charged linkers (e.g., phosphorothioate, phosphorodithioate, etc). The primers or probes of the invention may be also modulated

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using a label that can directly or indirectly provide a detectable signal. Examples of the label are radioisotopes, fluorescent molecules, and biotin.

According to another aspect of the invention, there is provided an antibody that may bind
5 to a protein expressed by the target gene. The antibody may bind specifically to the protein, or a partial peptide having a binding domain that is specific to the protein. Such method may allow for the measurement of the protein expression level.

By "measurement of the protein expression level", it is meant to include any process of
10 determining the presence of protein expression and extent of the protein expression. In an embodiment, the proteins may be confirmed using an antibody that binds specifically to a protein, or a partial peptide having a binding domain that is specific to a protein. Examples of the assay methods available for measuring the protein expression level include Western blotting, enzyme linked immunosorbent assay (ELISA), radioimmunoassay (RIA),
15 radioimmunodiffusion, Ouchterlony immunodiffusion, rocket immunoelectrophoresis, histoimmunostaining, immunoprecipitation assay, complement fixation assay, and FACS, or a method using a protein chip, but are not limited thereto.

The measurement of the protein expression level may be achieved preferably using
20 ELISA. Various types of ELISAs include direct ELISA in which a labelled antibody immobilized onto a solid support is used to recognize an antigen, indirect ELISA in which a labelled antibody is used to recognize a captured antibody immobilized on a solid support which is complex with an antigen, direct sandwich ELISA in which an antibody is used to recognize an antigen captured by another antibody immobilized onto a solid
25 support, and indirect sandwich ELISA in which a secondary antibody is used to recognize an antibody which captures an antigen complexed with a different antibody immobilized onto a solid support. More preferably, an antibody is immobilized onto a solid support and is reacted with a sample to form an antigen-antibody complex, and then, a labelled antibody specific to the antigen of the antigen-antibody complex is allowed to capture the
30 antigen of the complex, followed by enzymatic colour development. Alternatively, an antibody specific to the antigen of the antigen-antibody complex is allowed to capture the antigen of the complex and then is recognized by a labelled secondary antibody, followed by enzymatic colour development. The formation of the complex of a marker protein with

an antibody may be quantitatively measured so as to detect the presence of a 2019-nCoV RNA or target gene in a biological sample.

In addition, in some embodiments, the measurement of the protein expression level may be achieved preferably using Western blotting with at least one antibody for the proteins. The entire proteins are selected from a sample, separated according to size by electrophoresis, transferred onto a nitrocellulose membrane, and reacted with an antibody to form an antigen-antibody complex. The expression level of the protein is measured by determining the quantity of the complex using a labelled antibody, thereby confirming radiation exposure or extent of the irradiation.

The detection method may be conducted by measuring the protein expression level in the normal control group and the biological sample. The expression levels of the RNA or protein may be represented by differences between absolute (e.g., $\mu\text{g}/\text{mL}$) or relative (e.g., relative intensity of signal) scales of the marker.

The term "antibody" as a term known in the art refers to a specialized immunoglobulin which is directed toward an epitope. The antibody used in the present invention refers to an antibody that binds specifically to a target of the 2019-nCoV. The antibody may be prepared using a target gene of the 2019-nCoV. Here, the 2019-nCoV target gene is cloned in an expression vector so as to obtain a protein encoded by the 2019-nCoV target gene. The antibody may be in the form of a polyclonal antibody, a monoclonal antibody, and a recombinant antibody, and in this regard, all the immunoglobulin antibodies fall within the range of the antibody of the present invention. The antibody is in a complete form composed of two full-length light chains and two full-length heavy chains. In addition, special antibodies such as humanized antibodies are among the antibodies of the present invention. The antibodies may be used to confirm expression of the protein in a biological sample according to the known assays in the art, such as ELISA, RIA, sandwich assay, Western blotting or Immunoblotting for a polyacryl gel.

Another aspect of the present invention provides a kit for diagnosis of a 2019-nCoV infection or exposure.

The kit according to the present invention may diagnose a 2019-nCoV infection or exposure by detecting or measuring the presence of a 2019-nCoV target gene; the expression levels of IGFBP5 gene, a 2019-nCoV target gene or the protein. The kit of the present invention includes an agent such as primers or probes (described in this application) for measuring the 2019-nCoV target gene or the protein, i.e., primers or probes binding specifically to RNA of the 2019-nCoV target gene, antibodies binding specifically to 2019-nCoV target gene, partial peptides having a binding domain that binds specifically to 2019-nCoV target gene, or a combination thereof. In addition, the kit may include one or more components, solutions, or devices suitable for an analysis method to measure the expression level of the 2019-nCoV target gene.

According to an embodiment, the kit of the present invention for radiation exposure diagnosis may be a microarray for radiation exposure diagnosis that can measure expression levels of protein or mRNA of the protein-coding gene. The microarray for radiation exposure diagnosis may be easily manufactured by one of ordinary skill in the art according to methods known in the art using the marker of the present invention. According to an embodiment, the microarray may include mRNA of the IGFBP5-coding gene or cDNA with sequence corresponding to a fragment of the marker gene as a probe attached to a substrate.

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When the kit of the invention may include all essential elements necessary for carrying out RT-PCR. The RT-PCR kit may include test tubes or other suitable containers, reaction buffers, deoxyribonucleotides (dNTPs), enzymes such as Taq-polymerase and reverse transcriptase, a deoxyribonuclease (DNase), RNase inhibitor, dEPC-water, sterile water, and so on, in addition to primers each of which is specific to the 2019-nCoV RNA target gene. Also, the kit may include a pair of primers specific to genes that are used as a quantitative control group.

According to another embodiment, the kit of the present invention may include antibodies binding specifically to the 2019-nCoV RNA target gene, substrates for immunological detection of antibodies, suitable buffer solutions, coloring enzymes or fluorescent-labeled secondary antibodies, or coloring substrates. The matrix may be a nitrocellulose membrane, a 96-well plate made of polyvinyl resin or polystyrene resin, a glass slide glass. The coloring enzyme may be peroxidase or alkaline phosphatase, and the

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fluorescent material may be FITC or RITC. The coloring substrate may be 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), o-phenylenediamine (OPD), or tetramethyl benzidine (TMB).

- 5 In another aspect of the invention, there is provided a method for diagnosing a 2019n-CoV infection or exposure, the method comprising: (a) detecting or measuring expression levels of an 2019-nCoV RNA target gene or the protein in a sample from a patient, such detection or measurement methods include using a primer and/or probe described herein; (b) comparing the detection or measured expression levels with that of a normal control
10 group; and (c) determining if the patient is infected or exposed to the virus when the detection levels or expression levels of the patient's sample are higher than those of the normal control group.

By "biological sample" or "sample from a patient", it is meant to refer to tissues, cells,
15 whole blood, sera, plasma, sputum, saliva, cerebrospinal fluid or urine.

In the figures:

- Fig. 1 shows the sequences of the COVID-19 virus from samples taken from Wuhan in GISAID;
- 20 Fig. 2 shows a schematic of the target region of COVID-19 compared to other coronaviruses;
- Fig. 3 shows a schematic of the primers flanking the target region of the COVID-19 sequence;
- Fig. 4 show a schematic of another set of primers flanking a second target region of the
25 COVID-19 sequence;
- Figs. 5a-5b shows the protocol used to perform gradient annealing temperature for the primers;
- Figs. 6a-6b shows the results of gradient annealing temperature for SARS-CoV-2 detection with different primers;
- 30 Fig. 7 shows the results of the primers at different annealing temperatures;
- Figs. 8a-8b show the in silico evaluation results of the nCoV(113) forward primer;
- Figs. 9a-9b show the in silico evaluation results of the nCoV(118) forward primer;
- Figs. 10a-10c show the in silico evaluation results of the nCoV(113) reverse primer (which may be used with the nCoV(118) forward primer;

Fig. 11 shows the in silico evaluation results of the nCoV(113) probe (which may be used with the nCoV(118) forward primer).

Five complete genomes of the Betacoronavirus/Wuhan were published on GISAID (www.gisaid.org) on 12 Jan 2020 (Fig. 1) with Accession IDs: EPI_ISL_402119, EPI_ISL_402120, EPI_ISL_402121, EPI_ISL_402123, and EPI_ISL_402124.

Based on an understanding of the COVID-19 genome and a previous study (Ruan *et al.* The Lancet, Vol. 361, May 2003, 179-1785) on the homology of SARS coronavirus (SARS-CoV-1) and other coronavirus, it was postulated that the NSP3 proteinase region in the COVID-19 (SARS-CoV-2) genome may provide a suitable target region that is unique to COVID-19 and distinct from the other coronaviruses (see Fig. 2). In SARS-CoV-1, this target region is named NSP1 and refer to the same region as NSP3 in SARS-CoV-2. Thus, several primers were prepared and synthesised to target the NSP3 proteinase region of COVID-19 as a means to accurately and quickly identify the presence of COVID-19 via RT-PCT.

A number of primers and probes were designed using the OLIGO design program, synthesised and tested. Fig. 3 shows a schematic of a possible first target region unique to COVID-19, the primers flanking the target region, the mutations, and the region where the TaqMan probe anneals.

Primers that may be used for the first target region in Fig. 3:

nCoV(113)F: TGTATACGTTGTCTTTGGAGCACA (SEQ ID No. 1), standard desalting
 nCoV(113)R: GGTTTTAGATCTTCGCAGGCAAG (SEQ ID No. 2), standard desalting
 nCoV(113)LO: (6FAM)5'-CCATTCCCTGCGCGTCCTC-3'(BHQ1), HPLC grade;
 CCATTCCCTGCGCGTCCTC (SEQ ID No. 3)
 nCoV(118)F: CCTGGTGTATACGTTGTCTTTGGAG (SEQ ID No. 9), standard desalting
 nCoV(126)F: ACCAAATACCTGGTGTATACGTT (SEQ ID No. 10)

Fig. 4 shows a schematic of a possible second target region unique to COVID-19 and the primers flanking the target region. From Fig. 4, it may be seen that the SARS-CoV-2 virus have a different sequence from the COVID-19 sequences and in the target region and on

either side of the flanking region. Thus, the primers nCoV(110) are able to bind selectively to COVID-19 over SARS-CoV-2.

Primers that may be used for the second target region in Fig. 4:

- 5 nCoV(110)F: TGATGTTGTTCAAGAGGGTGTTT (SEQ ID No. 11)
 nCoV(110)R: AATTGTCTGTTGGCACTTTTCTCA (SEQ ID No. 12)
 nCoV(110)LO: (6FAM)5'-CCATTCCCTGCGCGTCCTC-3'(BHQ1);
 CATTTTCAGTAGTGCCACCAGCCTT (SEQ ID No. 13)

10 **EXAMPLE 1**

PCR Protocol: nCoV detection with IC using Bio-Rad iTaq Universal Probes

1. PCR reagent

iTaq Universal Probes One-Step Kit

Cat#172-5141 (500 x 20uL rxns)

15 **2. Master Mix preparation**

Reaction vol (ul) = 25

	Description	25 ul/Rxn (ul)
<i>From Bio-Rad Kit</i>	iTaq universal probes reaction mix (2x)	12.5
	iScript reverse transcriptase	0.5
	Nuclease-free water	8.40
Primers & Probes from EDDC-Dx	nCoV (113)+IC Primer-Probe mix (FAM/HEX)	1.00
	IC-77 Template (1000 copies/uL)	0.10
Test Sample	RNA sample	2.50
	Total Volume	25

3. RT-PCR cycling

	conditions	Biorad CFX96	
Step	Temp (°C) net (sec)	Duration	No. of Cycle(s)
1	48	15 mins	1
2	95	2 min 30 s	1
3	95	20 secs	42
	59*	42 secs	

20 * Perform fluorescence data collection here.

4. Data Analysis

Bio-Rad CFX 96				
No.	Target	Dye	Channel	Threshold
1	nCoV	FAM	FAM	50
2	IC	HEX	HEX	50

EXAMPLE 2

5 Primer Probe mix worksheet

Mix name:	nCov with Internal Control			
Ver_Lot #:	DxD REF: FH001			
No. of rxn to make (25uL rxn):	220		No. of tubes	1
Vol of primer mix used (25uL PCR rxn):	1		Rxn per tube:	220
	Oligo Name:	Primer stock (uM)	Final Conc. In rxn (uM)	Vol used (uL)
Oligo 1	nCoV Forward Primer	100uM	0.200	11.00
Oligo 2	nCoV Reverse Primer	100uM	0.200	11.00
Oligo 3	nCoV FAM Probe	100uM	0.100	5.50
Oligo 4	Internal Control Forward Primer	100uM	0.200	11.00
Oligo 5	Internal Control Reverse Primer	100uM	0.200	11.00
Oligo 6	Internal Control HEX Probe	100uM	0.100	5.50
		(Water / TE 1x) to add		165
		Primer conc (uM) in tube		5

		Probe conc (uM) in tube	2.5
		Total vol of stock used	55.00
		Vol. per tube	220
Labels:		NOTE:	
nCoV with Internal Control		nCoV with Internal Control	
PP Mix			
DxD REF: FH001			
10-Feb-20		Operator 1	

EXAMPLE 3

The following sets out an example of carrying out a method of detecting a 2019-nCoV target gene in a sample according to an embodiment of the invention.

5 **1 Introduction**

The following is a worksheet for performing quantitative TaqMan[®] real time PCR for detection of 2019-novel coronavirus (2019-nCoV) viral RNA. An exogenous internal control is included for the purpose of checking PCR inhibition.

2 Worksheet ID

10 Name the worksheet ID as follows:

SOP-DX-01- _Date(YYMMDD)Operator's initial

Example: SOP-DX-01- _200210M

ID:

3 Operator details

Name of operator: _____ Date: _____

Signature of _____
operator: _____

4 Equipment

Equipment	Manufacturer, Model	Serial Number	Calibration/ Maintenance Due Date (DDMMYY)	Initial
Mini centrifuge with PCR tubes	TOMY, microONE			

Equipment	Manufacturer, Model	Serial Number	Calibration/ Maintenance Due Date (DDMMYY)	Initial
adapter				
Mini centrifuge with 1.5ml tube adapter	TOMY, microONE			
Centrifuge with plate adapter	Eppendorf, 5430R			
Vortex	Scientific Industries, Vortex-Genie 2, G- 560E			
Ice maker	Hoshizaki, FM-120EE			
Real time PCR instrument	Bio-Rad, CFX96			
4°C and -20°C Combi Freezer	Sanyo, MPR-414F			
-30°C Freezer	Sanyo, MDF-U333			
-80°C Freezer	Sanyo, MDF-U73V			
Liquid Handling Machine	Eppendorf, epMotion, P5073c			
Laminar Flow Hood	Gelman, HLF 120			

5 Pipettes

Pipette	Manufacturer, Model	Serial Number	Calibration/ Maintenance Due Date (DDMMYY)	Initial

Pipette	Manufacturer, Model	Serial Number	Calibration/ Maintenance Due Date (DDMMYY)	Initial
Finnpipette™ 0.2-2 µL	Thermo Scientific™, F1, 4641010			
Research Plus 0.5-10 µL	Eppendorf, 3120 000.020			
Research Plus 2-20 µL	Eppendorf, 3120 000.097			
Research Plus 10-100 µL	Eppendorf, 3120 000.046			
Research Plus 30-300 µL	Eppendorf, 3120 000.100			
Research Plus 100-1000 µL	Eppendorf, 3120 000.062			
Xplorer, single-channel, 5-100 µL	Eppendorf, 4861000.023			
ep Motion Dispensing tool, TS 50, 1.0 - 50 µL	Eppendorf, 960001010			
ep Motion Dispensing tool, TS 300, 20 - 300 µL	Eppendorf, 960001028			

6 Consumables

Items	Vendor	Catalogue Number	Lot Number	Expiry Date (DDMMYY)	Initial
10 µL XL filter tips	Biotix	M-0011- 9FC		N/A	
20 µL epTIPS LoRetention Dualfilter	Eppendorf	30077628		N/A	

Items	Vendor	Catalogue Number	Lot Number	Expiry Date (DDMMYY)	Initial
100 μ L epTIPS LoRetention Dualfilter	Eppendorf	30077644		N/A	
300 μ L epTIPS LoRetention Dualfilter	Eppendorf	30077636		N/A	
1000 μ L epTIPS LoRetention Dualfilter	Eppendorf	30077652		N/A	
50 μ L epTIPS Motion Reload Filter	Eppendorf	0030014430 -SP		N/A	
300 μ L epTIPS Motion Reload Filter	Eppendorf	0030014472 -SP		N/A	
1.5 mL DNA LoBind tube	Eppendorf	0030108.05 1		N/A	
Multiplate™ 96-Well PCR Plates, low profile, unskirted, clear	Bio-Rad	MLL9601		N/A	
Microseal® 'B' PCR Plate Sealing Film, adhesive, optical	Bio-Rad	MSB1001		N/A	
8-well Optical Strip Caps	Stratagene, Agilent	STR_40142 5		N/A	

7 Reagents

Reagents	Vendor	Catalogue Number	Lot Number	Expiry Date (DDMMYY)	Initial
iTaq Universal Probes One-Step Kit	Bio-Rad	172-5141			

Reagents	Vendor	Catalogue Number	Lot Number	Expiry Date (DDMMYY)	Initial
nCoV+IC primer-probe mix	EDDC in-house	N/A			
Internal Control template, 1000 cp/μL	EDDC in-house	N/A			
UltraPure Distilled Water, DNase/RNase-Free	Invitrogen, Thermo Fisher Scientific	10977-015			

8 RNA Samples

EDDC Internal Sample ID	Lot Number	Total Volume Required (μL)	Initial

Note: Print this page as required if more samples are used.

Positive control	Lot Number	Total Volume Required (μL)	Initial
CoV 100 cp/uL			

5 9 Important information before starting experiment

- Switch on the CFX96 instrument and make sure it is properly synced with the computer.
- Perform all work in the laminar flow hood:
 - a. Switch on the UV lamp for 20mins to surface sterilize the hood.
 - 10 b. Switch off the UV lamp and switch on the blower and light.
 - c. Wipe the hood with 70% isopropyl alcohol.

- d. Wipe the surfaces of materials with 70% isopropyl alcohol before placing into the hood, eg pipettes, filtered tip boxes, 96-well plate holder, containers containing 1.5 mL DNA LoBind tubes, low profile 96-well plates, 8-well strip caps etc
- 5 e. Place a new small biohazard bag into the glass beaker for waste collection.
- Switch on epMotion P5073c (if necessary):
 - a. Wipe the working surfaces with 70% isopropyl alcohol.
 - b. Wipe the surfaces of materials with 70% isopropyl alcohol before placing into the epMotion eg dispensing tools, filtered tip boxes, 96-well plate
 - 10 holder, 1.5mL tube holder etc
 - c. Place a new small biohazard bag into the waste container.
 - d. Set up the protocol to be used according to manufacturer's manual.
 - Thaw all reagents and samples at 4°C.
 - Maintain thawed reagents and samples on a cold block or on ice until ready to be used.
- 15 **10 Preparation of PCR run**
- Briefly vortex to mix all reagent tubes, followed by pulse spin. Repeat for sample tubes and return all tubes to a cold block or on ice.
 - Prepare the PCR master mix using the table below in a 1.5 mL DNA LoBind tube on a
 - 20 cold block or on ice. Excess volume for half a reaction is included to make up for loss during reagent transfers.

PCR master mix components:

Components	Volume/ reaction (μ L)	_____ reactions	Date prepared (DDMMYY)	Initial
iTaq universal probes reaction mix (2x)	12.5			
iScript reverse transcriptase	0.5			
nCoV+IC primer-probe mix	1			
Internal Control template 1000 cp/ μ L	0.1			
Nuclease-free water	2.9			

Components	Volume/ reaction (μ L)	_____ reactions	Date prepared (DDMMYY)	Initial
Total volume	22.5			

- Briefly vortex the PCR master mix tube and pulse spin the contents. Repeat if air bubbles are present in the solution. Place the PCR master mix tube on a cold block or on ice.
- 5
- Prepare the unskirted low profile PCR plate to the desired number of wells and place it on a 96-well rack. The unskirted low profile PCR plate may be cut to size depending on the number of wells required.
 - Using the Xplorer multiple dispense mode, aliquot 22.5 μ L of PCR master mix into each well of the low profile PCR plate, as specified in the following PCR well layout.
- 10
- Add 2.5 μ L of RNA samples into each respective wells, followed by positive controls and lastly, nuclease-free water as no template control.

PCR well layout:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

- 15
- Seal the low profile 96-well plate with optical strip caps. If a full 96-well plate is utilised, seal the whole plate with a piece of adhesive film.
 - Briefly centrifuge the 96-well plate to remove air bubbles and to bring all the liquid to the bottom of each well. Use centrifuge with plate adapter for a full 96-well plate or

mini centrifuge with PCR tube adaptor for 1 or 2 columns of the 96-well plate. Repeat until there are no air bubbles present.

- Load the 96-well plate into CFX96 real time PCR instrument and clean the strip caps or adhesive film with Kimwipes before starting the run.
- 5 • Run the PCR on the CFX96 real time PCR instrument using the run parameters below:

Step	Temperature (°C)	Duration	No. of Cycle(s)
cDNA synthesis	48	15 mins	1
Denaturation	95	2 min 30 secs	1
PCR amplification	95	20 secs	42
	59*	42 secs	

* Perform fluorescence data collection here.

- Select the target dye channel as follows:

No.	Target	Dye	Channel
1	nCoV	FAM	FAM
2	IC	HEX	HEX

Start time of PCR run (00:00 AM/PM)	End time of PCR run (00:00 AM/PM)	Date (DDMMYY)	File name	Initial

- 10 • After the PCR run has finished, remove the 96-well plate from the instrument and disposed it into the biohazard waste bin.

11 Data analysis

- Select only 1 channel at a time, go to “Settings”, select “baseline threshold” and under “User defined”, manually set the threshold to 50.

No.	Target	Channel	Threshold
1	nCoV	FAM	50
2	IC	HEX	50

- Compute the Ct value and check that each positive amplification has a typical sigmoidal curve.

12 Abnormal Occurrences / Conclusion

- 5 List any abnormal occurrences (state if none occurred) and write a final conclusion in the box below:

Operator Print Name: _____

Operator Signature: _____ Date: _____

Reviewer Print Name: _____

Reviewer Signature: _____ Date: _____

EXAMPLE 4

- 10 Mutations in the viral genome can render the oligonucleotides to lose its binding to the target, causing false negatives in a small fraction of strains.

An enhancer primer, 118F, was created to enhance the detection sensitivity and minimize the false negative rates for SARS-CoV-2.

- 5 With reference to YiJun Ruan, et al. The Lancet Vo. 361. May 2003 1779-1785, NSP3 proteinase gene of 2019-nCoV also known as SARS-CoV-2 (this is the same region as NSP1 in SARS-CoV-1) was selected as the target region for the design of primers and probes that are less prone to future mutations.
- 10 The following example provides the use of an enhancer primer, to enhance the sensitivity of the kit for the detection of SARS-CoV-2, the cause of COVID.

- An enhancer was added to the kit, and compared with the kit without the enhancer. 1ul of water in the kit with the nCoV113 primers alone was replaced with 1ul of enhancer,
- 15 per tube.

- Four cases (1-4 below) were detected demonstrating a great increase in strength of the signal, as shown by a fall in Ct value and four new diagnoses of COVID-19 were made by adding the enhancer which were 'not detected' when tested without the enhancer. Details
- 20 are shown in Table 1 below.

Table 1: Ct values of kit without and with the enhancer in the testing of new subject samples

Cases	Ct values without enhancer	Ct values with enhancer	Effect
1	38.72	30.60	Stronger by -8 Cts
2	36.31	27.10	Stronger by -9 Cts
3	35.22	26.92	Stronger by -8 Cts
4	37.33	28.27	Stronger by -9 Cts
5	not detected	34.9	'New case'
6	not detected	35.14	'New case'
7	not detected	31.33	'New case'
8	not detected	34.54	'New case'

- 25 In addition, four further patients which had been diagnosed with the kit without enhancer, had additional samples submitted where the Ct values dropped significantly

(became stronger), when they would have been expected to increase (to become weaker) as the patients recovered clinically. The results are shown in Table 2.

Table 2: Ct values of kit with and without enhancer of previously tested samples

Cases	Ct values without enhancer	Ct values with enhancer	Effect
1	29.85	20.59	Stronger by -9 Cts
2	37.75	31.99	Stronger by -6 Cts
3	37.63	29.96	Stronger by -8 Cts
4	39.19	32.59	Stronger by -7 Cts

5

The data in Tables 1 and 2 demonstrate the clinical value of adding the enhancer for the continued and comprehensive detection of COVID-19 infected cases.

As time goes by, further mutations are expected, which will require similar enhancers.

- 10 The 118F enhancer (or forward primer) nCoV(118)F has the following sequence:
(5'-3')CCTGGTGTATACGTTGTCTTTGGAG,(SEQ ID No. 9),Standard desalting (IDT)

Example Concentrations with the enhancer 118F

Method 1- Separate tubes

- 15 A separate tube of 118F is prepared at concentration of 1.25uM (25X).
The final concentration of 118F is 0.05uM by adding 1uL of 1.25uM 118F into each 25uL RT-qPCR reaction volume.

Method 2- Inclusion of 118F into the premix in Example 2

- 20 Add an appropriate volume of 118F (higher concentration) to 113F/R/P, IC F/R/P at concentration of 1.25uM (25X).
The final concentration of 118F is 0.05uM in each 25uL RT-qPCR reaction volume.

Both methods to prepare the enhancer solution may be used in Example 3 above.

25

EXAMPLE 5

Gradient annealing temperature was performed on the primer sets (nCoV(110) and nCoV(113)) described above. The protocol used is shown in Figs. 5a and 5b. Briefly, a master mix containing 0.50 ul of iScript advanced reverse transcriptase (BioRad), 12.50 ul

of iTaq universal probes reaction mix (2x) (Buffer containing hot-start Taq DNA polymerase, dNTPs, MgCl₂, enhancers, stabilisers, passive dyes and ROX) (Biorad), 8.75 ul of water and 2.50 ul of a test RNA sample. The SARS-CoV-2 synthetic plasmid was used as the test sample.

5

0.50 ul of a 10uM solution of each of the three primer sets (forward and reverse primers) and 0.25 ul of a 10 uM solution of the corresponding FAM probe was added to the master mix.

Step	Temp (°C) net (sec)	Duration	No. of Cycle(s)
1	48	15 mins	1
2	95	2 mins 30s	1
3	95 51.5-60.5*	20s 42s	42

10

* Perform gradient temperature and fluorescence data collection here.

The annealing temperature was performed at 60.5°C, 60.0°C, 59.0°C, 57.2°C, 55.1°C, 53.3°C, 52.1°C, and 51.5°C.

15

The results are shown in Figs 6a-6b for nCov(110) and nCoV(113) respectively. Fig. 7 shows the Ct values of SARS-CoV-2 as the test sample with the primers at different annealing temperatures and is also shown in Table 3.

Table 3: Ct values of the primers at different annealing temperatures

Annealing Temp. (°C)	Ct value	
	Set 110	Set 113
60.0	30.68	30.32
59.0	30.67	29.56
57.2	30.80	30.19
55.1	31.34	29.45

53.3	32.20	30.12
------	-------	-------

From these results, it may be seen that the nCoV(113) primer set produce stronger fluorescence signal (at least double) compared to the nCov(110) primer set. The nCov(110) and nCoV(113) primers performed the best at an annealing temperature (T_a) of 59.0°C and 55.1°C respectively.

EXAMPLE 6

The kit containing the nCoV(113) primers was put into clinical service at a hospital to validate the kit's specificity and accuracy.

10

An inhouse comparison was made at the hospital with the NPHL 'N' gene assay and the kit of the examples above. The kit in particular with the nCoV(113) primers gave a cCt lower than the NPHL assay by 2-3 Ct units. Detailed results are shown below. Further a blinded comparison with the NPHL assay was also conducted.

15

Clinical experience:

8,755 samples were tested, from approximately 5,900 patients. Most were nasopharyngeal swabs in UTM; 500ul was extracted with the Nuclisens EasyMAG system (Biomeriux) and eluted in 55uL.

20

576 positive samples were detected from 95 patients (many repeats were sent as discharge from the hospital required two negative samples) using the kit with the nCoV(113) primers.

25

Of these 95 patients, 21 of the 95 patients were confirmed 'positive' by the NPHL assay, early on in the outbreak. 66 of the 95 patients were repeatedly positive with the kit with the nCoV(113) primers (not tested by NPHL). All were believed, clinically. 8 of the 95 patients had only one positive sample.

30

The NPHL 'N' gene kit were used to re-test 11 RNA preps, using the original RNA, stored at -80 °C. The RNA had been frozen and thawed, some of it a few times for other evaluations. These 11 were made up of the 8 with only one positive sample mentioned

above, and 3 extras (numbered 9, 10, and 11) added in to generate a little comparative Ct data. Results are shown below in Table 4 (ND = not detected).

Table 4: Ct values of samples tested with nCoV(113) primers and NPHL N gene assay

	Sample Type	Kit with nCoV(113) primers	Kit with nCoV(113) primers Ct Value	NPHL N gene Ct value
1	NPS	DET.	36.17	39.74
2	NPS	DET.	37.56	ND
3	NPS	DET.	35.62	40.36
4	NP/throat swab	DET.	34.77	38.09
5	Stool	DET.	34.1	38.67
6	NP/throat swab	DET.	36.54	38.03
7	Sputum	DET.	32.15	34.95
8	ETTA	DET.	32.2	33.64
9	NPS	DET.	23.98	25.26
10	Nasal swab	DET.	19.18	20.94
11	Nasal swab	DET.	29.3	31.17

5 DET.: Means detected

Ten of the eleven samples were also ‘detected’ by the NPHL kit, all with weaker (i.e. larger) Ct values compared to that obtained with the kit containing the nCoV(113) primers. Case (or sample) 2 in Table 4, was not detected by the NPHL assay. The original Ct value
 10 was 37.56, so it would not be surprising for repeatedly thawed RNA to degrade a little and thus not be detected in subsequent analysis. This patient (case 2) had been sampled on day 21 after symptom onset, and had a positive contact history with another proven COVID case, hence it is believed he/she was a true case although it has not been verified by a different PCR system.

15

In addition, for the first 81 cases, all were found to be epidemiologically connected with previous cases, or with a travel history from Hubei province, China. The initial 7,240 samples were tested without any apparent false positives and validated the effectiveness and specificity of the kit containing the nCoV(113) primers. Epidemiology for subsequent
 20 cases was not recorded.

In addition, a specificity check was conducted (100% were 'not detected' for SARS-CoV-2) against a panel of 24 RNA preps from an external quality assurance scheme containing;

Ten x MERS-CoV

Influenza H3N2 A/Brisbane/117/2018

5 Influenza H1N1 A/Sydney/134/2018

Influenza H9N2 A/chicken/Myanmar/433/2016

Influenza H3N2 A/Brisbane/1028/2017

Influenza H7N9 A/Guangdong/17SF003/2016

Influenza H3N2 A/Brisbane/1028/2017

10 Influenza H3N2 A/Perth/1001/2018

Influenza H1N1 A/Victoria/2040/2018

Influenza B/Maryland/15/2016

Influenza B/Townsville/8/2016

Two x Coronavirus OC43

15 Two x Coronavirus NL63

The kit with the nCoV(113) primers only returned a positive result with the COVID-19 virus and not the other RNA preps tested above.

20 This validates the accuracy and specificity of the kit containing the nCoV(113) primers. In particular, the kit shows 100% specificity and gave lower Ct values compared with the NPHL 'N' gene assay.

EXAMPLE 7

25 An in silico evaluation of the nCoV(113) and nCoV(118) primers against 268011 sequences of the COVID-19 virus was done to evaluate the effectiveness of the primers against the COVID-19 virus and the various mutations in the target region of the primers. The results are shown in Figs. 8a-8b, 9a-9b, 10a-10c and 11. The sequences of the cases shown in these Figures are only the region where the primer hybridised with the sample
30 DNA and not the entire sequence. The case numbers for each primer is specific for that primer.

For the nCoV(113) primers, it was determined that 263276 of 268011 sequences were covered (98.23%). 4735 of 268011 sequences missed (1.77%) with at least 1 mismatch
35 (2799 forward, 1917 reverse, 38 probe), 3523 of 268011 sequences missed (1.31%) with at least 1 mismatch in 3' region (2560 forward, 954 reverse, 16 probe), and 878 of 268011

sequences missed (0.33%) with at least 2 mismatches in 3' region (877 forward, 1 reverse, 0 probe).

For the nCoV(118)F primers (nCov(118)F and nCoV(113)R), it was determined that
5 199736 of 268011 sequences were covered (74.53%). 68275 of 268011 missed (25.47%)
with at least 1 mismatch (66413 forward, 1917 reverse, 38 probe), 1116 of 268011 missed
(0.42%) with at least 1 mismatch in 3' region (147 forward, 954 reverse, 16 probe) and 1 of
268011 missed (0.00%) with at least 2 mismatches in 3' region (0 forward, 1 reverse, 0
probe).

10

The in silico results show that a kit containing the nCoV(113) primers detected the
COVID-19 sequences with very high accuracy and supports the comparative Ct data in
Example 6. The use of the nCoV(118)F primer alone with the nCoV(113)R primer gave a
lower detection rate than the nCoV(113) pair of primers. However, the addition of the
15 nCoV(118) primer to the pair of nCoV(113) primes provided improved detection
capability to the kit, in particular for mutants in the region where the nCoV(113)F primer
pairs with the virus strain.

20

The examples above show that the nCoV(113) primers were effective at providing a means
to detect and distinguish the COVID-19 virus quickly and in a cost-effective manner. The
early detection may help to control the spread of the virus at an early stage and prevent it
from escalating out of control. The nCoV(118) primer alone while not as effective as the
nCoV(113) primer, provides enhanced detection of the COVID-19 mutations in
combination with the nCoV(113) primers.

25

Whilst there has been described in the foregoing description preferred embodiments of the
present invention, it will be understood by those skilled in the technology concerned that
many variations or modifications in details of design or construction may be made without
departing from the present invention.

30