

## METHODS AND KITS FOR IMPROVED PCR

### CROSS REFERENCE TO RELATED APPLICATION

**[0001]** This application claims the priority to Singapore patent application No. 10202003067V, filed 1 April 2020, the contents of which are incorporated herein by reference.

### FIELD

**[0002]** The present disclosure relates generally to PCR methods and kits for detection of biological targets such as biological infectious agents.

### BACKGROUND

**[0003]** The following discussion of the background to the invention is intended to facilitate an understanding of the present invention only. It should be appreciated that the discussion is not an acknowledgement or admission that any of the material referred to was published, known or part of the common general knowledge of the person skilled in the art in any jurisdiction as at the priority date of the invention.

**[0004]** Most PCR detection kits are sold as vials of reagents that are sensitive to temperature and require cold chain logistics for shipping and delivery. During the current COVID-19 pandemic some shipping and delivery has slowed down or stopped making it difficult to ship PCR detection kits. Further, such shipment is often expensive and difficult particularly to remote locations. It is not unusual for epidemics to start in remote locations where it may be difficult to ship reagents at cold temperatures.

**[0005]** SARS-CoV-2 is a coronavirus that emerged from Wuhan, China, in Dec 2019, leading to global human-to-human transmission and spread of a disease that leads to symptoms such as fever, cough, fatigue, and shortness of breath (Huang et al., The Lancet (2020)). In response to the spread of SARSCoV-2, the World Health Organisation (WHO) has since declared that COVID-19—the respiratory disease caused by SARS-CoV-2—be declared a Public Health Emergency of International concern, and thereafter, a pandemic (WHO Director-General's opening remarks at the media briefing on SARS-COV-2 – 11 March). This virus has since resulted in supply chain breakdowns and has had an unprecedented effect on the global economy.

**[0006]** Severe cases of SARS-CoV-2 infection may lead to acute respiratory distress syndrome, organ failure, sepsis, septic shock and death (Johns Hopkins University and Medicine: Corona virus Resource Centre. (2020)). As of 1 April 2020, 859,877

confirmed cases and 42,342 deaths across 203 countries and regions – increasing at a rate of over 30,000 cases per day have been reported (Johns Hopkins University and Medicine: Corona virus Resource Centre. (2020)). However, the current reported COVID-19 incidence numbers are still considered to be an under-estimate and are expected to continue rising as the virus has not yet been contained, and several countries still do not have sufficient diagnostic tests or testing capabilities.

**[0007]** The WHO has associated severe COVID-19 with the development of severe pneumonia, sepsis, and septic shock (WHO. Clinical management of severe acute respiratory infection when novel coronavirus (2019-nCoV) infection is suspected 2020). Approximately 20% of COVID-19 cases are severe and have been associated with sepsis, with the remaining 80% of cases being mild or uncomplicated (WHO. Report of the WHO-China Joint Mission on Coronavirus Disease 2019 (SARS-COV-2). 2020). Out of the severe cases, 25.4% become critical and develop multiple organ failure and septic shock, 49% of whom die.

**[0008]** The severity of COVID-19 is associated with poorer outcomes and a high mortality rate. COVID-19 has been shown to disproportionately adversely affect those who are older and those with co-morbidities as summarised in Table 1.

**[0009]** Table 1: Comorbidities of COVID-19 (WHO. Report of the WHO-China Joint Mission on Coronavirus Disease 2019 (SARS-COV-2). (2020)

| <b>Comorbidity</b>          | <b>Case fatality rate</b> |
|-----------------------------|---------------------------|
| Cardiovascular disease      | 13.2%                     |
| Diabetes                    | 9.2%                      |
| Hypertension                | 8.4%                      |
| Chronic Respiratory Disease | 8.0%                      |
| Cancer                      | 7.6%                      |
| No pre-existing conditions  | 0.9%                      |
| <b>AGE GROUPS</b>           |                           |
| >80 years old               | 21.9%                     |
| 70-79 years old             | 8.0%                      |
| 60-69 years old             | 3.6%                      |
| 50-59 years old             | 1.3%                      |
| 40-49 years old             | 0.4%                      |
| <40 years old               | <1.0%                     |

**[0010]** As SARS-CoV-2 is associated with severe outcomes such as organ failure and death; early identification and treatment of SARS-CoV-2 patients are critical to improving clinical outcomes. Qin-Sun et al. reported that patients who received treatment and interventions earlier in the disease progression presented better clinical outcomes with mortality rate decreased (Sun et al., *Annals of Intensive Care*. 10(33). (2020)).

**[0011]** On 23 Jan 2020, Singapore reported its first imported case of SARS-CoV-2. Since then, there have been 923 confirmed cases of SARS-CoV-2 infection, as of 31 March 2020. Of these cases 519 (56%) of these cases were confirmed to be imported cases, and 404 cases were locally transmitted (44%), according to the Singapore Ministry of Health (MOH (Singapore). Official update of SARS-COV-2 situation in Singapore as of 31 March 2020).

**[0012]** Most cases are reported with a mild and uncomplicated disease progression. Severe cases were associated with increased age and the presence of comorbidities. As of 31 March 2020, Singapore had 423 active cases (46% of all cases), of whom 401 are stable and 22 patients in critical condition, in addition to three deaths; 500 cases have recovered and have subsequently been discharged.

**[0013]** After nearly two months of battling the disease, Singapore reported its first two deaths, followed a week later by a third death. All fatality cases were over the age of 60 years and both had pre-existing medical conditions. However, one of the cases were presented to the hospital in critical condition, emphasising the need for more effective diagnostic tests that would allow for patients to be diagnosed reliably and early prior to deterioration.

**[0014]** As Singapore currently has an ageing population, one of the highest rates of diabetes in the world, with cardiovascular disease and cancer as a leading cause of death, **resulting in Singapore's population currently having a significant number of people** susceptible to serious complications of COVID-19. Therefore, early and prompt detection of SARS-CoV-2 would greatly aid national quarantine and treatment programmes, preventing the further epidemiological spread of the disease.

**[0015]** There is no internationally recognised standardised method to diagnose SARS-CoV-2. There are, however, four primary methods used: (a) complete genetic sequencing (>24 hours turnaround, high accuracy); (b) RT-PCR (1-6 hours turnaround, most cost-effective method and the gold standard in infectious disease testing; (c) chest computed tomography (CT) (>1 hour turnaround, often only reliable in late-stage infection,

no longer used as a diagnostic criterion); (Mossa-Basha, et al. Radiology. Doi: <https://doi.org/10.1148/radiol.2020200988> (2020)) (d) chest X-ray (>1 hour turnaround; returned 50% false-negative results in Singapore) (Young BE, et al. SARS-COV-2 Singapore Case series. (2020)).

**[0016]** RT-PCR is the preferred test for viral infectious diseases such as COVID-19 caused by RNA viruses, as this method offers high sensitivity rates while also being high-throughput. However, the current available RT-PCR tests have been shown to produce false-negative results, twice in some cases (CNA. New Coronavirus found in Japan evacuees who initially tested negative. (2020)) when retested and even with true-positive CT results. This could be due to factors such as poor specimen collection practices, specimen contamination, and low viral load present in the specimen. RNA samples are especially vulnerable to degradation due to RNase.

**[0017]** A shortage of test kits has been reported in several countries including the United States and Japan, either not having enough tests kits or having reached maximum throughput of their existing diagnostic capabilities. Besides testing suspected patients, the Singapore Ministry of Health announced during a press conference that they will step up precautionary efforts by screening all patients with pneumonia (500-600 a week).

**[0018]** Printing of oligonucleotides of nucleic acid libraries onto microarrays are known and many arrays are available such as those from Agilent Affymetrix or Illumina. The libraries are printed into plates using spotting robots also known as gridders, printers, printing robots or microarray robots. Generally, each spot or location printed on a microarray contains many copies of an identical oligonucleotide probes of a known sequence. It is then possible to determine whether that sequence is present in DNA of a test sample.

**[0019]** There exists a need to have alternatives PCR methods and kits and alleviate at least one of the aforementioned problems.

## SUMMARY

**[0020]** A method for making ready to use PCR detection kits, and the resulting kits being able to be stored and transported at room temperature is envisaged. In some embodiments a cost-effective, high-throughput diagnostic kit that overcomes the drawbacks of current RT-PCR tests is envisioned.

**[0021]** Accordingly, an aspect of the invention refers to a method of forming a PCR

detection kit comprising: printing a first nucleotide probe, a first nucleotide forward primer and a first nucleotide reverse primer in a printer buffer together into each of a plurality of first wells within one plate; printing a second nucleotide probe, a second nucleotide forward primer and a second nucleotide reverse primer in a printer buffer together into each of a plurality of second wells adjacent the first wells within the one plate; drying the wells; and vacuum sealing the plate.

**[0022]** According to another aspect there is a polymerase chain reaction (PCR) detection kit comprising dried and sealed first nucleotide probe, first nucleotide forward primer and first nucleotide reverse primer pre-printed together into each of a plurality of first wells within one plate; second nucleotide probe, second nucleotide forward primer and second nucleotide reverse primer pre-printed together into each of a plurality of second wells adjacent the first wells within the one plate.

**[0023]** According to another aspect there is a method for detecting the presence of an infectious agent in a sample comprising:

isolating nucleic acid from the sample;

adding PCR reagents and the isolated nucleic acid into a first well having first nucleotide probe, first nucleotide forward primer and first nucleotide reverse primer pre-printed and dried therein;

adding PCR reagents and the isolated nucleic acid into a second well having second nucleotide probe, second nucleotide forward primer and second nucleotide reverse primer pre-printed and dried therein;

run a PCR reaction; and

determine whether the sample has the infectious agent present.

**[0024]** Other aspects and features of the present invention will become apparent to those of ordinary skill in the art upon review of the following description of specific embodiments of the invention in conjunction with the accompanying figures.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

**[0025]** In the figures, which illustrate, by way of non-limiting examples only, embodiments of the present invention,

**[0026]**        **Figure 1:** Embodiments of a substrate layout with primers and probes printed and dried onto a 96 well plate. Plates in these embodiments are available in either configuration A or configuration B; both correspond to the same Legend C.

**[0027]**        **Figure 2:** Overview of the workflow from reconstituting primers and probes to loading the source plate for printing.

**[0028]**        **Figure 3:** Overview workflow of diluting forward primers, reverse primers, and probes for the RdRP gene assay to be printed into the 96-well plates

**[0029]**        **Figure 4:** A schematic of an embodiment of the detection system in use.

**[0030]**        **Figure 5:** An overview of the master mix to be used with an embodiment.

#### DETAILED DESCRIPTION

**[0031]**        Throughout this document, unless otherwise indicated to the contrary, the terms “comprising”, “consisting of”, “having” and the like, are to be construed as non-exhaustive, or in other words, as meaning “including, but not limited to”.

**[0032]**        Furthermore, throughout the document, unless the context requires otherwise, the word “include” or variations such as “includes” or “including” will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

**[0033]**        Unless defined otherwise, all other technical and scientific terms used herein have the same meaning as is commonly understood by a skilled person to which the subject matter herein belongs.

**[0034]**        According to various embodiments, there is a PCR detection kit comprising dried and sealed nucleotide probe, nucleotide forward primer and nucleotide reverse primer present in a plurality of wells within one plate.

**[0035]**        According to various embodiments, there is a method of forming a PCR detection kit comprising printing a first nucleotide probe, a first nucleotide forward primer and a first nucleotide reverse primer in a printer buffer together into each of a plurality of first wells within one plate; printing a second nucleotide probe, a second nucleotide forward primer and a second nucleotide reverse primer in a printer buffer together into each of a plurality of second wells adjacent the first wells within the one plate; drying the wells; and

vacuum sealing the plate.

**[0036]** By pre-printing and drying the primers and probes into the plate wells themselves as compared to packing them in tubes for cold chain logistics, the kit is able to be compact allowing more tubes for other reagents to be packed into the same box, catering to more patient samples per test kit. This saves on shipment space and costs per test and allows the plates to be shipped by any form of transport to any location without refrigeration required.

**[0037]** As used herein the term 'printing', 'print', 'pre-printing' or 'pre-printed' refers to mechanically placing the primers and probes into wells on the plate using spotting robots also known as gridders, printers, printing robots or microarray robots. The advantage of printing is that there is less room for human error or contamination and the quality control is higher as each well is printed with a measured amount with a very high level of reproducibility.

**[0038]** In various embodiments, the first nucleotide probe, first nucleotide forward primer and first nucleotide reverse primer detect a first target sequence of an infectious disease and the second nucleotide probe, second nucleotide forward primer and second nucleotide reverse primer detect a second target sequence of the same infectious disease. Having two sets of primers and probes pre-printed and dried into the plate wells allows for two different target sequences from the same infectious agent to be detected at the same time to increase the accuracy of any test and reduce false results.

**[0039]** In various embodiments, the nucleotide primers and probe are DNA, however any other nucleic acids known in the art such as linked nucleic acid would also be suitable provided the nucleic acids are stable at room temperature when dried in the wells. In various embodiments, the plurality of wells are 2 or more, 4 or more, 6 or more 8 or more, 16 or more, 32 or more, 96 or more or any number of wells that allows testing of 2 or more infectious agent target nucleic acids. In various embodiments, the buffer is a TE buffer (Tris, EDTA buffer).

**[0040]** In various embodiments, the first nucleotide probe comprises SEQ ID NO. 5, the first nucleotide forward primer comprises SEQ ID NO. 3, the first nucleotide reverse primer comprises SEQ ID NO. 4, the second nucleotide probe comprises SEQ ID NO. 8; the second nucleotide forward primer comprises SEQ ID NO. 6; and the second nucleotide reverse primer comprises SEQ ID NO. 7.

**[0041]** In various embodiments, the kit is made to detect a first and second target sequence of an infectious disease. In various embodiment the first target sequence to be detected comprises a fragment of the RNA expressing RNA-dependent RNA polymerase RdRp in SARS-Cov2 coronavirus set forth in SEQ ID NO 1:

CTTGTCACACCGTTTCTATAGATTAGCTAATGAGTGTGCTCAAGTATTGAGTGAAATGG  
TCATGTGTGGCGGTTCACTATATGTTAAACCAGGTGGAACCTCATCAGGAGATGCCAC  
AACTGCTTATGCTAATAGTGTTTTAAACATTTGTCAAGCTGTCACGGCCAATGTTAATG  
CACTTTTATCTACTGATGGTAACAAAAT.

**[0042]** In various embodiments, the first forward primer used for the detection of SEQ ID NO 1 comprises the nucleotide sequence set forth in SEQ ID NO. 3:

GTGARATGGTCATGTGTGGCGG, wherein the R may be G or A. For example, AAA or AGA. Preferably the R in nucleotide sequence set forth in SEQ ID NO. 3 is an A.

**[0043]** In various embodiments, the first reverse primer used for the detection of SEQ ID NO 1 comprises the nucleotide sequence set forth in SEQ ID NO. 4:

CARATGTTAAAAACACTATTAGCATA, wherein the R may be G or A. For example AAA or AGA. Preferably the R in nucleotide sequence set forth in SEQ ID NO. 4 is an A.

**[0044]** In various embodiments, the first probe used for the detection of SEQ ID NO 1 comprises the nucleotide sequence set forth in SEQ ID NO. 5: -56-  
FAM/CAGGTGGAACCTCATCAGGAGATGC/3BBQ650/

**[0045]** In various embodiments, the second target sequence to be detected comprises a fragment of the RNA expressing the Envelope protein in SARS-Cov2 coronavirus as set forth in SEQ ID NO. 2:

ATGAGTACGAACTTATGTACTCATTCGTTTCGGAAGAGACAGGTACGTTAATAGTTAAT  
AGCGTACTTCTTTTTCTTGCTTTTCGTGGTATTCTTGCTAGTTACACTAGCCATCCTTACT  
GCGCTTCGATTGTGTGCGTACTGCTGCAATATTGTTAACGTGAGTCTTGTAACCTT  
CTTTTACGTTTACTCTCGTGTTAAAAAT.

**[0046]** In various embodiments, the second forward primer used for the detection of SEQ ID NO 2 comprises the nucleotide sequence set forth in SEQ ID NO. 6:  
ACAGGTACGTTAATAGTTAATAGCGT.

**[0047]** In various embodiments, the second reverse primer used for the detection of SEQ ID NO 2 comprises the nucleotide sequence set forth in SEQ ID NO. 7:  
ATATTGCAGCAGTACGCACACA.

**[0048]** In various embodiments, the second probe used for the detection of SEQ ID NO 2 comprises the nucleotide sequence set forth in SEQ ID NO. 8: /56-FAM/ACACTAGCCATCCTTACTGCGCTTCG/3BBQ650/.

**[0049]** In various embodiments, the plurality of first wells comprises a row of wells for detection of a first target nucleic acid sequence and the plurality of second wells comprise a row of wells for detection of a second target nucleic acid sequence..

**[0050]** This allows detection in many samples with increased accuracy as two different target sequences from the same infectious agent are able to be detected at the same time in multiple samples to increase the accuracy and throughput of any test.

**[0051]** In various embodiments, each well of the row of wells in the plurality of first wells is printed with the first nucleotide probe comprising SEQ ID NO. 5; the first nucleotide forward primer comprising SEQ ID NO. 3; and the first nucleotide reverse primer comprising SEQ ID NO. 4; and in each well of the row of wells in the plurality of second wells is printed with the nucleotide probe comprising SEQ ID NO. 8; the nucleotide forward primer comprising SEQ ID NO. 6; and the nucleotide reverse primer comprising SEQ ID NO. 7.

**[0052]** In various embodiments, the method further comprises printing at least one well in the plate with a control nucleotide probe, a control nucleotide forward primer and a control nucleotide reverse primer in a printer buffer prior to drying and sealing the plate.

**[0053]** In various embodiments, the control nucleotide probe comprises SEQ ID NO. 12; the control nucleotide forward primer comprises SEQ ID NO. 10 and the control nucleotide reverse primer comprises SEQ ID NO. 11.

**[0054]** In various embodiments, the target sequence to be detected is an internal Control sequence comprising MS2 Phage Gene. In various embodiments the internal control target sequence to be detected is the nucleotide sequence set forth in SEQ ID No. 9:

CGGTCGTTTTAACTCGACTGGGGCCAAAACGAAACAGTGGCACTACCCCTCTCCGTA  
TTCACGGGGGGCGTTAAGTGTACATCGATAGATCAAGGTGCCTACAAGCGAAGTGG  
GTCATCGTGGGGTCGCGGTACGAGGAGAAAGCCGTTTCGGCTTCTCCCTCGACG  
CACGCTCCTGCTACAGCCTCTCCCTGTAAGCCAAAACCTTGACTTACATCGAAGTGCC  
GCAGAACGTTGCGAACCGGG.

**[0055]** In various embodiments, the control forward primer used for the detection of SEQ ID NO 9 for the internal control comprises the nucleotide sequence set forth in SEQ ID NO. 10: TGGCACTACCCCTCTCCGTATTAC.

**[0056]** In various embodiments, the control reverse primer used for the detection of SEQ ID NO 9 for the internal control comprises the nucleotide sequence set forth in SEQ ID NO. 11: GTACGGGCGACCCACGATGAC.

**[0057]** In various embodiments, the forward primer used for the detection of SEQ ID NO 9 for the internal control comprises the sequence set forth in SEQ ID NO. 12: /56-JOEN/CACATCGATAGATCAAGGTGCCTACAAGC/3BHQ 1/.

**[0058]** In various embodiments, the wells are dried in an oven. In various embodiments, the wells containing the nucleotide primers and probe are dried in an oven. In various embodiments, the wells containing the nucleotide primers and probe are dried in an oven after they have been printed into the wells. In various embodiments, the primers and probes in the wells are dried at a temperature that does not denature or degrade the nucleotide sequences. In various embodiments the primers and probes in the wells may be dried at a temperature that may be 30°C or less. In various embodiments, the primers and probes in the wells may be freeze dried.

**[0059]** In various embodiments, the plate comprises a 96 well plate. This allows the test to be used with common equipment used in the art such as centrifuges, PCR equipment such as racks and cyclers as well as other equipment used.

**[0060]** In various embodiments, the method further comprises packing a plurality of the plates into one box. In various embodiments, a plurality of plates may include 2 or more, 4 or more, 6 or more, 8 or more plates, or any number of plates convenient for shipping. In various embodiments, packing 6 plates into one kit is able to compact more tubes into the same box, catering to more patient samples per test kit (up to 276 patients tested per kit of 6 x 96-well plates), saving on shipment space and costs per test and allowing the plates to be shipped by any form of transport to almost any location they may be needed. The same kits can be stored without refrigeration.

**[0061]** According to various embodiments, there is a polymerase chain reaction (PCR) detection kit comprising dried and sealed first nucleotide probe, first nucleotide forward primer and first nucleotide reverse primer pre-printed together into each of a plurality of first wells within one plate; second nucleotide probe, second nucleotide forward

primer and second nucleotide reverse primer pre-printed together into each of a plurality of second wells adjacent the first wells within the one plate.

**[0062]** By pre-printing and drying the first and second primers and probes into the plate wells in the kit, as compared to packing them in tubes that require cold chain logistics, the kit is able to compact more tubes into the same box, catering to more patient samples per test kit. This saves on shipment space and costs per test and allows the kit to be shipped by any form of transport to any location without refrigeration required. Further, the kit has a very high level of accuracy overall as the two sets of pre-printed primers and probes dried into the plate wells are more consistent, have low chance of contamination and allows for two different target sequences from the same infectious agent to be detected at the same time. This reduces the chance of false results in each test.

**[0063]** In various embodiments, the nucleotide primers and probe are DNA, however any other nucleic acids known in the art such as linked nucleic acid LNA or any other nucleic acid that would be suitable to be stable at room temperature when dried in the wells. In various embodiments, the plurality of wells is 2 or more, 4 or more, 6 or more, 8 or more, 16 or more, 32 or more, 96 or more or any number of wells that allows testing of 2 or more samples. In various embodiments, each well in the plate comprises dried and sealed nucleotide probe, nucleotide forward primer and nucleotide reverse primer pre-printed into the well.

**[0064]** In various embodiments, the first nucleotide probe comprises SEQ ID NO. 5, the first nucleotide forward primer comprises SEQ ID NO. 3, the first nucleotide reverse primer comprises SEQ ID NO. 4, the second nucleotide probe comprises SEQ ID NO. 8; the second nucleotide forward primer comprises SEQ ID NO. 6; and the second nucleotide reverse primer comprises SEQ ID NO. 7. In various embodiments, the primers and probes are as disclosed herein above.

**[0065]** In various embodiments, the plurality of first wells comprise a first row of wells for detection of a first target nucleic acid sequence and the plurality of second wells comprise a second row of wells for detection of a second target nucleic acid sequence.

**[0066]** In various embodiments, the primers and probe in the first row of wells is for detection of a first target nucleic acid sequence of an infectious agent and the primers and probe in the second row of wells is for detection of a second target nucleic acid sequence of the same infectious agent. This allows for detection in many samples with increased accuracy as two different target sequences from the same infectious agent may be

detected in multiple samples at the same time to increase the throughput and accuracy of any test.

**[0067]** In various embodiments, each well of the first row is pre-printed with the first nucleotide probe comprising SEQ ID NO. 5; the first nucleotide forward primer comprising SEQ ID NO. 3; and the first nucleotide reverse primer comprising SEQ ID NO. 4; and in each well of the second row is pre-printed with the second nucleotide probe comprising SEQ ID NO. 8; the second nucleotide forward primer comprising SEQ ID NO. 6; and the second nucleotide reverse primer comprising SEQ ID NO. 7. In various embodiments, the primers and probes are as disclosed herein above. In various embodiments, the first primers and probes in the first row of wells is for detection of a first target nucleic acid sequence SEQ ID NO. 1 of the infectious agent SARS CoV2 and the second primers and probes in the second row of wells is for detection of a second target nucleic acid sequence SEQ ID NO. 2 of the infectious agent SARS CoV2.

**[0068]** In various embodiments, the method further comprises at least one well in the plate pre-printed with a control nucleotide probe, a control nucleotide forward primer and a control nucleotide reverse primer.

**[0069]** In various embodiments, the control nucleotide probe comprises SEQ ID NO. 12; the control nucleotide forward primer comprises SEQ ID NO. 10 and the control nucleotide reverse primer comprises SEQ ID NO. 11. In various embodiments, the control primers and probes are as disclosed herein above.

**[0070]** In various embodiments, the plate comprises a 96 well plate. In various embodiments, the plate of the kit comprises a 96 well plate.

**[0071]** In various embodiments, the kit further comprises a plurality of plates. In various embodiments, the plurality of plates may include 2 or more, 4 or more, 6 or more, 8 or more plates, or any number of plates convenient for shipping. In various embodiments, the kit comprises 6 plates in one kit allowing the kits to be compact, fitting more tubes into the same box, catering to more patient samples per test kit (up to 276 patients tested per kit of 6 x 96-well plates), saving on shipment space and costs per test and allowing the plates to be shipped by any form of transport to almost any location they may be needed.

**[0072]** In various embodiments, the kit further comprises a second kit containing PCR reagents for use with the pre-printed primers and probes in the plurality of wells within the plate.

**[0073]** PCR reagents require refrigeration and cold chain logistics. Having a second kit containing PCR reagents allows the second kit that requires refrigeration, to be shipped independently to the kit comprising the dried and sealed pre-printed primers and probe. In various embodiments, the PCR reagents of the second kit may be used for PCR detection for any primers or probes. This allows kit developers preparing kits for a sudden outbreak of an infectious disease by a new infectious agent to quickly develop, prepare and ship kits for detecting the presence of the infectious agent as the only component that needs to be adapted is the kit comprising the dried and sealed pre-printed first and second primers and probe suitable to detect the presence of the new infectious agent. In various embodiments, remote locations may already have stock of the second kit such that only the kit comprising the dried and sealed pre-printed first and second primers and probe needs to be shipped to the remote location to facilitate rapid delivery and detection of the new infectious agent in samples. This is particularly relevant where epidemics or pandemics result in the slowing of transport and shipment of refrigerated goods to affected areas.

**[0074]** According to various embodiments, there is a method for detecting the presence of an infectious agent in a sample comprising:

isolating nucleic acid from the sample;

adding PCR reagents and the isolated nucleic acid into a first well having first nucleotide probe, first nucleotide forward primer and first nucleotide reverse primer pre-printed and dried therein;

adding PCR reagents and the isolated nucleic acid into a second well adjacent the first well having second nucleotide probe, second nucleotide forward primer and second nucleotide reverse primer pre-printed and dried therein;

run a PCR reaction; and

determine whether the sample has the infectious agent present.

**[0075]** Using wells having a first and second nucleotide probe, nucleotide forward primer and nucleotide reverse primer pre-printed and dried therein minimises the number of reagent handling steps needed in reaction set-up in the testing laboratory. Sample handling and pipetting steps are reduced, lowering the critical risks of sample degradation, cross-contamination, and critically, human error.

**[0076]** In various embodiments the samples may be patient samples from patients

at risk of being or suspected to be infected with the infectious agent. In various embodiments the sample may be a nasopharyngeal or oropharyngeal swab, or other suitable body fluid sample extracted for *in vitro* analysis. In various embodiments the nucleic acid isolated from the sample is RNA. In various embodiments the nucleic acid isolated from the sample is DNA.

**[0077]** In various embodiments, the primers and probe in the first well is for detection of a first target nucleic acid sequence of an infectious agent and the primers and probe in the second well is for detection of a second target nucleic acid sequence of the same infectious agent. This allows for two different target sequences from the same infectious agent to be detected at the same time to increase the accuracy of any test.

**[0078]** In various embodiments, the infectious agent may be any pathogenic organism that causes an infectious disease. In various embodiments, the infectious agent may be any pathogenic microorganism such as bacteria, virus, parasite or fungus.

**[0079]** In various embodiments, the infectious agent is a virus. In various embodiments, the infectious agent or the virus may be a single-stranded (ss) or a double-stranded (ds), RNA or DNA virus including those that have reverse transcriptase and those that do not have reverse transcriptase.

**[0080]** In various embodiments, the infectious agent is a virus. In various embodiments, the virus is an RNA virus. In various embodiments, the RNA virus comprises single-stranded (ss) RNA virus including those employing sense strands such as Coronaviruses, Picornaviruses, or Togaviruses and those employing antisense strands such as Orthomyxoviruses, or Rhabdoviruses. In various embodiments, the RNA virus comprises double-stranded (ds) RNA viruses such as Reoviruses. In various embodiments, the RNA virus comprises single-stranded (ss) reverse transcriptase RNA viruses such as Retroviruses. In various embodiments, the RNA virus comprises double-stranded (ds) reverse transcriptase RNA viruses such as Hepadnaviruses.

**[0081]** RNA is very sensitive to degradation making it difficult to detect infections caused by RNA viruses. Using the wells having nucleotide probes, nucleotide forward primers and nucleotide reverse primers pre-printed and dried therein minimises the number of reagent handling steps needed in reaction set-up in the testing laboratory. Sample handling and pipetting steps are reduced, lowering the critical risks of RNA degradation in the sample. The detection can also be done closer to the patient as the kits can be shipped without refrigeration so the samples need not be sent off to distant laboratories in times of

crisis again lowering the critical risks of RNA degradation in the sample.

**[0082]** In various embodiments, the infectious agent is a coronavirus. In various embodiments, the virus is a coronavirus. In various embodiments, the RNA virus is a coronavirus. In various embodiments, the single-stranded (ss) RNA virus is a coronavirus.

**[0083]** In various embodiments, the infectious agent is a SARS CoV-2 coronavirus. In various embodiments, the virus is a SARS CoV-2 coronavirus. In various embodiments, the RNA virus is a SARS CoV-2 coronavirus. In various embodiments, the single-stranded (ss) RNA virus is a SARS CoV-2 coronavirus. In various embodiments, the Coronavirus is a Severe Acute Respiratory Syndrome Coronavirus 2 (SARS CoV-2).

**[0084]** In various embodiments, the first nucleotide probe comprises SEQ ID NO. 5 the first nucleotide forward primer comprises SEQ ID NO. 3 the first nucleotide reverse primer comprises SEQ ID NO. 4; the second nucleotide probe comprises SEQ ID NO. 8; the second nucleotide forward primer comprises SEQ ID NO. 6; and the second nucleotide reverse primer comprises SEQ ID NO. 7. In various embodiments, the primers and probes are as disclosed herein above.

**[0085]** In various embodiments, the first well comprises the nucleotide probe comprising SEQ ID NO. 5; the nucleotide forward primer comprising SEQ ID NO. 3; and the nucleotide reverse primer comprising SEQ ID NO. 4 pre-printed and dried therein and a second well comprises the nucleotide probe comprising SEQ ID NO. 8; the nucleotide forward primer comprising SEQ ID NO. 6; and the nucleotide reverse primer comprising SEQ ID NO. 7 pre-printed and dried therein. In various embodiments, the primers and probes are as disclosed herein above.

**[0086]** In various embodiments, the method further comprises having a well comprising a negative and/or a positive control.

**[0087]** In various embodiments, the method further comprising spiking the sample with a nucleic acid sequence unrelated to the infectious diseases; adding PCR reagents and the spiked isolated nucleic acid into a third well having a control nucleotide probe, a control nucleotide forward primer and a control nucleotide reverse primer pre-printed and dried therein and detecting the presence of the nucleic acid sequence unrelated to the infectious diseases in the third well as a positive control.

**[0088]** In various embodiments, each sample may be processed with three

controls, with the aim to reduce false negative and false positive results. In various embodiments, an internal control containing whole MS2 phage (an RNA virus; safe at BSL-1 standards) is spiked into the sample prior to sample processing, and acts as a co-purification RNA extraction control as well as for detection of any PCR inhibition; this prevents false negatives in SARS-CoV-2 infection diagnosis reporting. Thereafter, a Positive PCR Control and a Negative PCR Control may act as quality checkpoints for a qRT-PCR run.

**[0089]** In various embodiments, the control nucleotide probe comprises SEQ ID NO. 12; the control nucleotide forward primer comprises SEQ ID NO. 10 and the control nucleotide reverse primer comprises SEQ ID NO. 11. In various embodiments, the control primers and probes are as disclosed herein above.

**[0090]** By pre-printing RT-PCR DNA primers and probes into the 96-well array plates themselves as compared to packing them in tubes for cold chain logistics, the kit is able to compact more tubes for other reagents into the same box, catering to more patient samples per test kit (up to 276 patients tested per kit of 6 x 96-well plates), saving on shipment costs per test and allowing the plates to be shipped by any form of transport.

**[0091]** In an epidemic or pandemic it may be advantageous to reduce air freight shipment, costs, and freight transit time, considering that flights or transport to the effected area or globally may be affected by such a crisis; cold-chain shipping is especially limited under such circumstances and in-demand in a period of such a crisis where cold-chain freight services may be delayed or cancelled to the area or worldwide.

**[0092]** In various embodiments the kit may be used with a second kit containing standard RT-PCR reagents or reagents specifically designed for use with the pre-printed RT-PCR DNA primers and probes into the 96-well array plates.

**[0093]** By minimising the number of reagent handling steps needed in reaction set-up in the testing laboratory, sample handling and pipetting steps are reduced, lowering the critical risks of sample degradation, cross-contamination, and critically, human error.

**[0094]** Kits containing pre-printing RT-PCR DNA primers and probes into the 96-well array plates thus may be able to cater to more patients per volume shipped via cold chain freight services and reduces the chances for losses in perishables should cold chain services be disrupted and delayed.

**[0095]** The kit eliminates manual pipetting of excessive PCR reagents that may create the potential for technical errors, poor inconsistencies by virtue of their labour-intensive nature and thus poor result reproducibility. Using reagents pre-printed and prepared in the 96-well reaction plates offers ease-of-use and minimises hands-on time, which are essential for improved laboratory through-put as well as yielding consistent and timely results.

**[0096]** The kit design reduces the number of freeze-thaw cycles that reagents are subject to; critical reagents in Acu-Corona™ 2.0 Kit A (requiring -20°C storage conditions) are aliquoted in small volumes in multiple vials to reduce degradation due to freeze-thaw cycles.

**[0097]** Referring to Figure 1, in one embodiment the plate layout has two sets of nucleotide probe, nucleotide forward primer and nucleotide reverse primer, wherein both sets are directed to the detection of a nucleotides expressing different proteins unique to the same infectious agent or organism to allow detection of that infectious agent or organism. In this particular example the first set is directed to SEQ ID No. 1 a fragment of RNA expressing RNA-dependent RNA polymerase RdRp in SARS-Cov2 coronavirus and the second set is directed to SEQ ID No. 2 a fragment of RNA expressing the Envelope protein in SARS-Cov2 coronavirus.

**[0098]** In various embodiments, Acu-Corona™ 2.0 aims to aid in the early and prompt detection of SARS-COV-2, supporting national quarantine and treatment programmes, thereby preventing the further epidemiological spread of the disease.

**[0099]** In various embodiments the sequence detected comprises a fragment of the RNA expressing RNA-dependent RNA polymerase RdRp in SARS-Cov2 coronavirus set forth in SEQ ID NO 1:

CTTGTCACACCGTTTCTATAGATTAGCTAATGAGTGTGCTCAAGTATTGAGTGAAATGG  
TCATGTGTGGCGGTTCACTATATGTTAAACCAGGTGGAACCTCATCAGGAGATGCCAC  
AACTGCTTATGCTAATAGTGTTTTTAACATTTGTCAAGCTGTCACGGCCAATGTTAATG  
CACTTTTATCTACTGATGGTAACAAAAT

**[00100]** In various embodiments the forward primer used for the detection of SEQ ID NO 1 comprises the sequence set forth in SEQ ID NO. 3:  
GTGARATGGTCATGTGTGGCGG

**[00101]** In various embodiments the reverse primer used for the detection of SEQ ID

NO 1 comprises the sequence set forth in SEQ ID NO. 4:

CARATGTTAAASACACTATTAGCATA

**[00102]** In various embodiments the probe used for the detection of SEQ ID NO 1 comprises the sequence set forth in SEQ ID NO. 5: -  
FAM/CAGGTGGAACCTCATCAGGAGATGC/3BBQ650/

**[00103]** In various embodiments the sequence detected comprises a fragment of the RNA expressing the Envelope protein in SARS-Cov2 coronavirus as set forth in SEQ ID NO. 2:  
ATGAGTACGAACTTATGTACTCATTCGTTTCGGAAGAGACAGGTACGTTAATAGTTAAT  
AGCGTACTTCTTTTCTTGCTTTCTGCTGATTCTTGCTAGTTACACTAGCCATCCTTACT  
GCGCTTCGATTGTGTGCGTACTGCTGCAATATTGTTAACGTGAGTCTTGTAACCTT  
CTTTTACGTTTACTCTCGTGTTAAAAAT.

**[00104]** In various embodiments the forward primer used for the detection of SEQ ID NO 2 comprises the sequence set forth in SEQ ID NO. 6:  
ACAGGTACGTTAATAGTTAATAGCGT

**[00105]** In various embodiments the reverse primer used for the detection of SEQ ID NO 2 comprises the sequence set forth in SEQ ID NO. 7:  
ATATTGCAGCAGTACGCACACA

**[00106]** In various embodiments the probe used for the detection of SEQ ID NO 2 comprises the sequence set forth in SEQ ID NO. 8: /56-  
FAM/ACACTAGCCATCCTTACTGCGCTTCG/3BBQ650/

**[00107]** In various embodiments the Internal Control comprises (MS2 Phage) Gene. In various embodiments the internal control detected is the sequence set forth in SEQ ID No. 9:  
CGGTCGTTTTAACTCGACTGGGGCCAAAACGAAACAGTGGCACTACCCCTCTCCGTA  
TTCACGGGGGGCGTTAAGTGTCACATCGATAGATCAAGGTGCCTACAAGCGAAGTGG  
GTCATCGTGGGGTCGCGGTACGAGGAGAAAGCCGGTTTCGGCTTCTCCCTCGACG  
CACGCTCCTGCTACAGCCTCTTCCCTGTAAGCCAAAACCTTGACTTACATCGAAGTGCC  
GCAGAACGTTGCGAACCGGG

**[00108]** In various embodiments the forward primer used for the detection of SEQ ID NO 9 for the internal control comprises the sequence set forth in SEQ ID NO. 10:

TGGCACTACCCCTCTCCGTATTAC

**[00109]** In various embodiments the reverse primer used for the detection of SEQ ID NO 9 for the internal control comprises the sequence set forth in SEQ ID NO. 11:  
GTACGGGCGACCCCACGATGAC

**[00110]** In various embodiments the forward primer used for the detection of SEQ ID NO 9 for the internal control comprises the sequence set forth in SEQ ID NO. 12: /56-JOEN/CACATCGATAGATCAAGGTGCCTACAAGC/3BHQ\_1/

**[00111]** According to various embodiments, there is a method of forming a PCR detection kit comprising printing a nucleotide probe, a nucleotide forward primer and a nucleotide reverse primer together in a printer buffer into a plurality of wells within one plate; drying the wells; and vacuum sealing the plate.

**[00112]** Referring to Figure 2, in one embodiment the nucleotide probe, the nucleotide forward primer and the nucleotide reverse primer are first mixed together in a TE printing buffer (Tris, EDTA buffer).

**[00113]** Examples

**[00114]** Acu-Corona™ 2.0 (Acumen Diagnostics Pte. Ltd. Singapore)

**[00115]** Acu-Corona™ 2.0 is a non-automated, *in vitro* qualitative diagnostic assay based on real-time reverse transcription and polymerase chain reaction (RT-PCR) and that detects SARS-CoV-2 RNA in RNA samples extracted from patient specimens. It consists of Acu-Corona™ 2.0 Kit A (contains an RT-PCR enzyme mix, nuclease-free water, internal control, and positive control) and Acu-Corona™ 2.0 Kit B (contains 96-well array plates with printed and dried RT-PCR primers and dual-labelled hydrolysis probes, and plate seals).

**[00116]** Acu-Corona™ 2.0 requires RNA samples extracted and purified through an RNA extraction and purification system. RNA sample elutes should be extracted from swabs (nasopharyngeal and oropharyngeal) and should be of high quality as per the laboratory's standards.

**[00117]** Acu-Corona™ 2.0 uses RNA samples extracted from patient swab (nasopharyngeal and oropharyngeal) samples to diagnose SARS-CoV-2 infection. One-step reverse transcription and quantitative polymerase chain reaction (qRT-PCR) targets

two SARS-CoV-2 ss-RNA sequences. First, viral ss-RNA is transcribed to cDNA, followed by PCR amplification and detection of probe dye intensities during qRT-PCR.

**[00118]**      **Acu-Corona™** 2.0 is designed to employ pre-printed PCR arrays in 96-well format which allows up to 46 patient samples per every 1.5-hr qRT-PCR run, supporting mass diagnostics efforts cost-effectively. Each patient sample is processed with three controls, with the aim to reduce false negative and false positive results. An internal control containing whole MS2 phage (an RNA virus; safe at BSL-1 standards) is spiked into the neat patient sample prior to sample processing, and acts as a co-purification RNA extraction control as well as for detection of any PCR inhibition; this prevents false negatives in SARS-CoV-2 infection diagnosis reporting. Thereafter, a Positive PCR Control and a Negative PCR Control act as quality checkpoints for the qRT-PCR run.

**[00119]**      **Acu-Corona™** 2.0 is a one-step reverse transcription and real-time quantitative polymerase chain reaction (one-step qRT-PCR) qualitative assay for the detection of SARS-CoV-2 ss-RNA from patient samples. Patient samples for **Acu-Corona™** 2.0 are RNA extracted from patient swabs (nasopharyngeal and oropharyngeal). These samples do not require culturing prior to the start of **Acu-Corona** use.

**[00120]**      The test is performed with 96-well plates pre-printed with the necessary primers and probes needed for the PCR reactions. The assay uses fluorescence RT-PCR technology to target specific primers and probes at two target sites unique to the SARS-CoV-2 genome, in addition to one set of primers and probe targeting a site unique to the internal control material added to the patient sample. The oligonucleotide probes consist of **attached 5'-end fluorophores as reporters and 3'-end probe quenchers**. Real-time multiplex monitoring of quantitative fluorescence intensities allows real time qualitative detection of SARS-CoV-2 in specimens. Refer to Figure 1 for the plate layout used in **Acu-Corona™** 2.0.

**[00121]**      **Acu-Corona™** 2.0 Internal Control (IC) comprises of the MS2 phage (whole bacteriophage containing RNA as its genetic material; BSL-1) that is spiked into the neat patient sample (1:100) and co-extracted and follow through the RT-PCR, ie. eluted together with total RNA (host and viral, if any). Internal Control RNA is transcribed and amplified during one-step RT-qPCR. Thus, the IC would act as an RNA extraction and purification quality control marker, as well as detect any potential PCR inhibition for every sample tested. The test is considered invalid if no IC RNA is detected finally based on RT-PCR results, which may suggest a failure in the extraction step or the presence of PCR inhibitors in the RNA sample.

**[00122]** Table 2: Raw Materials and Specifications of the Kit together with the reagent kit.

| Kit | Component                            | Manufacturer      | CAT. #        | Specs                                              |
|-----|--------------------------------------|-------------------|---------------|----------------------------------------------------|
|     | Kit A box                            | CH Paper          | Non-catalogue | Art Card, laminated                                |
|     | Taqman Fast Virus 1-step Master Mix  | Life Technologies | 444436        | 1-Step qRT-PCR primer-probe detection reaction mix |
|     | Distilled water (UltraPure)          | Life Technologies | 10977015      | Membrane-filtered water                            |
|     | MS2 phage internal control           | Zeptomatrix       | 0810274       | 5x10e10 pfu/mL                                     |
|     | PBS buffer                           | Thermofisher      | PD8117        | Cell-culture/PCR grade; to be diluted to 1X        |
|     | SARS-CoV-2 E gBlock                  | IDT               | Non-catalogue | 250 ng                                             |
|     | SARS-CoV-2 RdRp gBlock               | IDT               | Non-catalogue | 250 ng                                             |
|     | MS2 phage gBlock                     | IDT               | Non-catalogue | 250 ng                                             |
|     | TE buffer                            | IDT               | 11-05-01-13   | PCR grade; to be diluted to 1X                     |
|     | Micro Tubes 2.0ml skirted, with caps | Labcon            | 3621-875      | Sterility recommended, not a requirement           |
|     | Micro Tubes 0.5ml skirted, with caps | Labcon            | 3641-875      | Sterility recommended, not                         |

|  |                                           |                   |               |                                                               |
|--|-------------------------------------------|-------------------|---------------|---------------------------------------------------------------|
|  |                                           |                   |               | a requirement                                                 |
|  | Laser Cryo-Babies 0.94 x 0.50"            | DivBio            | LCRY-2380     | Cryo-labels - withstand up to - <b>190°C and</b> condensation |
|  | Laser Cryo-Tags 3.0 x 2.0"                | DivBio            | LCRY-3020     | Cryo-labels - withstand up to - <b>190°C and</b> condensation |
|  | Kit B box                                 | CH Paper          | Non-catalogue | Art Card, laminated                                           |
|  | 96-well plate, fully skirted, low profile | Agilent           | 401490        | 96-well plates for use with Agilent AriaDx                    |
|  | 96-well LC480 (hard-shell) plates         | Bio-Rad           | HSR9905       | 96-well plates for use with LC480                             |
|  | HSP-96,WHT/CLR 50/BX                      | Bio-Rad           | HSP9601       | 96-well plates for use with Bio-Rad CFX96                     |
|  | ABI MicroAmp Fast Optical 96-Well Plate   | Life Technologies | 4366932       | 96-well plates for use with ABI 7500 Fast                     |

**[00123]**      Acu-Corona™® 2.0 96-well array RT-qPCR plates are printed and dried with RT-qPCR reaction DNA primers and probes. Printing of these ingredients are performed by the Agilent Bravo Automated Liquid Handling Platform (Agilent Cat# G5563AA).

**[00124]**      Acu-Corona™® 2.0 primers and probes are printed and dried into the provided PCR array plates; this gives comparative clinical advantages over traditional RT-PCR test methods.

**[00125]** the production procedures for printing PCR oligo DNA primers and PCR oligo DNA probes into 96-well plates for Acu-Corona™® 2.0 test for the detection of SARS-CoV-2.

**[00126]** Material required:

**[00127]** Reagents Required per 60 Target Plates

**[00128]** a. TE buffer (BU-0002-P)

**[00129]** b. RdRP\_SARSr-F2 Sequence, SEQ ID NO. 3

**[00130]** c. RdRP\_SARSr-R1 Sequence , SEQ ID NO. 4

**[00131]** d. RdRP\_SARSr-P2 Sequence , SEQ ID NO. 5

**[00132]** e. E\_Sarbeco\_F1 Sequence , SEQ ID NO. 6

**[00133]** f. E\_Sarbeco\_R2 Sequence, SEQ ID NO. 7

**[00134]** g. E\_Sarbeco\_P1 Sequence , SEQ ID NO. 8

**[00135]** h. MS2-Fw-1 Sequence (SN-000010), SEQ ID NO. 10

**[00136]** i. MS2-Rv-1 Sequence (SN-000011), SEQ ID NO. 11

**[00137]** j. MS2-Probe-1 Sequence (SN-000012), SEQ ID NO. 12

**[00138]** Equipment Required

**[00139]** a. 10 mL pipette

**[00140]** b. 1,200 µL micropipette

**[00141]** c. 300 µL micropipette

**[00142]** d. 100 µL micropipette

**[00143]** e. Plate centrifuge

**[00144]** f. Vortex machine

**[00145]** g. Tube racks for 1.5 mL tubes

**[00146]** Consumables Required**[00147]** a. 1.5 mL microcentrifuge tubes (12 pcs)**[00148]** b. Pipette tips for all above pipettes**[00149]** c. PCR foil seal strong (SN-CP-0002; 61 pcs)**[00150]** d. 0.2 mL PCR tubes

**[00151]** For each dried form of vendor vial, add x  $\mu$ L of TE buffer directly into the oligonucleotide tube (Primers and probe) ; **exact volume in  $\mu$ L is as**, according to the specifications sheet delivered with the corresponding primer / probe. Refer to Figure 3 for the overview workflow of diluting primers and probes for the RdRP gene assay. The **amount of TE buffer to aliquot to achieve 100  $\mu$ M should be mentioned** on the specification sheet (the specifications sheet will be delivered together with the primers and probes).

**[00152]** From the Seed Stock ("SS") vial that should contain the primer / probe in 100  $\mu$ M concentration, divide the amount of TE buffer in  $\mu$ L required by 20 and round down to the immediate whole number. This is the number of 0.2 mL microtubes to be prepared for the next step. Aliquot 20  $\mu$ L of the SS into each 0.2 mL microtube. Label all derived 0.2 mL microtubes as forward primer eg: "RdRP-F G1" for the forward primer, reverse primer eg: "RdRP-R G1" for the reverse primer, or probe eg: "RdRP-P G1" for the probe.

**[00153]** Store all reconstituted primers and probes in -20°C when not in use. They should be left out at room temperature for a maximum of three hours and should as best be kept away from direct light where possible.

**[00154]** Retrieve a first set of nucleotide probe, nucleotide forward primer and nucleotide reverse primer, for example "RdRP-F G1", "RdRP-R G1", "RdRP-P G1". A second set of nucleotide probe, nucleotide forward primer and nucleotide reverse primer, for example "E-F G2", "E-R G2", "E-P G2", an internal control set of nucleotide probe, nucleotide forward primer and nucleotide reverse primer, for example "MS2-F IC", "MS2-R IC" and "MS2-P IC" stocks – retrieve as many as needed for the planned number of plates to be printed within a 3-hour window (one source plate serves 60 target plates, sufficient for ten Acu-Corona™ 2.0 kits).

**[00155]** Fully thaw all these primers and probes before use.

**[00156]** Prepare 12 1.5 mL microcentrifuge tubes in a tube rack. Aliquot the following reagents in Table 3 into each of 1.5 mL tubes.

**[00157]** Table 3: Preparation of G1+IC Primers and Probes Mix (for each 1.5 mL tube; for 8 wells)

| Reagent                            | Volume for a single reaction | Volume for one 1.5 mL tube* (Aliquot this amt) |
|------------------------------------|------------------------------|------------------------------------------------|
| TE buffer                          | 1.97 $\mu$ L                 | 1,040.16 $\mu$ L                               |
| RdRP_SARSr F2 primer (100 $\mu$ M) | 0.12 $\mu$ L                 | 63.36 $\mu$ L                                  |
| RdRP_SARSr R1 primer (100 $\mu$ M) | 0.16 $\mu$ L                 | 84.48 $\mu$ L                                  |
| RdRP_SARSr Probe 2 (100 $\mu$ M)   | 0.04 $\mu$ L                 | 21.12 $\mu$ L                                  |
| MS2 Forward primer (100 $\mu$ M)   | 0.08 $\mu$ L                 | 42.24 $\mu$ L                                  |
| MS2 Reverse primer (100 $\mu$ M)   | 0.08 $\mu$ L                 | 42.24 $\mu$ L                                  |
| MS2 Probe (100 $\mu$ M)            | 0.05 $\mu$ L                 | 26.4 $\mu$ L                                   |
| Final reaction volume              | 2.5 $\mu$ L                  | 1,320.0 $\mu$ L                                |

\*One 1.5mL tube is for 480 reactions \* 1.1 = 528 reactions

**[00158]** Aliquot the following reagents in Table 4 into each of 1.5 mL tubes.

**[00159]** Table 4: Preparation of G2+IC Primers and Probes Mix (for each 1.5 mL tube; for 8 wells)

| Reagent                           | Volume for a single reaction | Volume for one 1.5 mL tube* (Aliquot this amt) |
|-----------------------------------|------------------------------|------------------------------------------------|
| TE buffer                         | 2.09 $\mu$ L                 | 1,103.52 $\mu$ L                               |
| E_Sarbeco F1 primer (100 $\mu$ M) | 0.08 $\mu$ L                 | 42.24 $\mu$ L                                  |
| E_Sarbeco R2 primer (100 $\mu$ M) | 0.08 $\mu$ L                 | 42.24 $\mu$ L                                  |
| E_Sarbeco Probe (100 $\mu$ M)     | 0.04 $\mu$ L                 | 21.12 $\mu$ L                                  |
| MS2 Forward primer (100 $\mu$ M)  | 0.08 $\mu$ L                 | 42.24 $\mu$ L                                  |
| MS2 Reverse primer (100 $\mu$ M)  | 0.08 $\mu$ L                 | 42.24 $\mu$ L                                  |
| MS2 Probe (100 $\mu$ M)           | 0.05 $\mu$ L                 | 26.4 $\mu$ L                                   |
| Final reaction volume             | 2.5 $\mu$ L                  | 1,320.0 $\mu$ L                                |

\*One 1.5mL tube is for 480 reactions \* 1.1 = 528 reactions

**[00160]** Mix the reagents for example via pulse vortex all the tubes on high speed for 10 seconds.

**[00161]** Seal and store the source plate.

**[00162]** Oligonucleotide printing

**[00163]** Gently remove the sealing foil from the source plate to avoid agitating the contents and begin printing. Once every 6 plates are printed, the program should pause to allow the operator to seal all 6 plates for centrifugation. Centrifuge all freshly printed plates at 1,000 g for 3 minutes. Every time the centrifugation is completed, gently retrieve the plates from the centrifuge, keep them upright without tilting them, and place them on a 96-well plate rack, and remove the plate seal very gently.

**[00164]** Drying

**[00165]** Place the plates into the drying oven, with the flap open at 100%, and dry at about 30°C.

**[00166]** Sealing

**[00167]** After the target plates have dried, seal the dried target plates with PCR Foil Seal Strong (material code CP-0002). For LC-SP plates, seal the plates with fresh optical strip caps (material code CP-0007) instead of the PCR foil seal strong films.

**[00168]** Pack each plate into the barrier foil pouches (material number PB-0003); seal the plates using the vacuum sealer machine. Store these plates in room temperature before packing/knitting and shipping.

**[00169]** By pre-printing RT-PCR DNA primers and probes into the 96-well array plates themselves as compared to packing them in tubes for cold chain logistics, Acu-Corona™ 2.0 is able to compact more tubes for other reagents into the same box, catering to more patient samples per test kit (up to 276 patients tested per kit of 6 x 96-well plates), saving on shipment costs per test.

**[00170]** Acu-Corona™ 2.0 is thus able to cater to more patients per volume shipped via cold chain freight services and reduces the chances for losses in perishables should cold chain services be disrupted and delayed.

**[00171]** Use of test kits

**[00172]** Acu-Corona™ 2.0 is a non-automated, in vitro, qualitative diagnostic assay based on one-step reverse transcription and quantitative PCR for the diagnosis of COVID-

19.

**[00173]** The sample type required for Acu-Corona™ 2.0 is RNA samples extracted and purified through a standard RNA extraction and purification system. RNA sample elutes may be extracted from patient swab (nasopharyngeal and oropharyngeal) **specimens and should be of high quality as per the laboratory's standards.**

**[00174]** Preferably test operators trained and authorised to use the Acu-Corona™ 2.0 for Applied Biosystems® 7500 Fast Real-Time PCR System may perform the test. Acu-Corona™ 2.0 4x Reaction Mix, Nuclease-Free Water, and Internal Control are non-toxic and non-hazardous. Any serious incident that has occurred in relation to Acu-Corona™ 2.0 should be reported to the manufacturer and the authority having jurisdiction where the Acu-Corona™ 2.0 was purchased or used. For more information, please consult the respective safety data sheet (SDS) from the product owner.

**[00175]** Acu-Corona™ 2.0 was developed, validated, and verified on the Applied Biosystems® 7500 Fast PCR System. It is currently also undergoing validation and verification testing on the Roche LightCycler® 480 Instrument II and Bio-Rad CFX96 platforms. By adopting these widely adopted systems, we reduce implementation barriers and make Acu-Corona™ 2.0 readily available for use globally.

**[00176]** Limit of Detection (LoD)

**[00177]** The LoD studies determine the lowest detection concentration of the SARS-CoV-2 at which approximately 95% of all replicates test positive. Due to the limitation of sample availability for this study, artificial synthetic SEQ ID NO 1 and SEQ ID NO. 2 was used as the template. The LoD was determined by testing serial dilutions of the positive sequence samples diluted in TE buffer. gBlock dsDNA SEQ ID NO 1 and SEQ ID NO. 2 **was synthesised by IDT and its synthesis yield was converted to copies/µl based on the molecular weight of the synthesised region.** Realtime RT-PCR assays were performed using Acu-Corona™ 2.0 reagents and consumables on the Applied Biosystems® 7500 Fast Real-time PCR instrument by two operators in one day.

**[00178]** A preliminary LoD concentration range was determined by testing triplicate samples. The approximate LoD was then identified by testing serial dilutions of the test stock with 22 replicates. Reactions that generated Ct values above the determined LoB were qualified as detected. Data are listed in Table 5a.

**[00179]** Table 5a: LoD results for SARS-CoV-2 RdRp gene and E gene; concentration is presented in RNA copies/ 20µl reaction

|                    | RdRp Gene  |            |           |          | E Gene     |            |           |          |
|--------------------|------------|------------|-----------|----------|------------|------------|-----------|----------|
| RNA concentration  | 500 copies | 100 copies | 50 copies | 5 copies | 500 copies | 100 copies | 50 copies | 5 copies |
| Positive/Total     | 22/22      | 20/22      | 10/22     | 0/22     | 22/22      | 22/22      | 22/22     | 5/55     |
| Mean Ct            | 33.1       | NA         | NA        | NA       | 36.5       | 36.4       | 38.3      | NA       |
| Standard deviation | 0.24       | NA         | NA        | NA       | 2.0        | 1.39       | 2.31      | NA       |

**[00180]** The LoD was determined based on the detection data and probit analysis as indicated in CLSI (2012) Evaluation of detection capability for clinical laboratory measurement procedures (EP17-A2). The 95% probability LoD is **116 copies/20 µl reaction for RdRP gene and 12.7 copies/20 µl reaction for E gene.**

**[00181]** The limit of detection analysis above can be used to determine the same for any primer and probe set pre-printed into the wells of plates as described herein above.

**[00182]** The limit of detection is the concentration of SARS-CoV-2 at which 95% of all replicates test positive. For this study, whole SARS-CoV-2 RNA from ATCC® VR-1986D™ (LN: 70035624; Concentration: 4730 genome copies/µL) was used as the PCR template. The RNA was extracted from live SARS-CoV-2 viruses and its copy number was determined by ATCC via ddPCR.

**[00183]** The LoD study was run by testing serial dilutions of the RNA template using 1x IDTE buffer as the diluent. Four dilution levels were made and for each dilution level, 20 replicates were tested. Reactions that generated Cq values below the determined Limit of Blank were qualified as detected.

**[00184]** The 95% LoD was then computed through a probit regression curve generated on Analyse-it. The data obtained are tabulated in Table 5b.

Table 5b: LoD results for RdRP and E gene assays of Acu-Corona® 2.0

|                     | RdRP Gene |       |        |         | E Gene |       |        |         |
|---------------------|-----------|-------|--------|---------|--------|-------|--------|---------|
| RNA Concentration   | 4.73      | 2.365 | 1.1825 | 0.59125 | 4.73   | 2.365 | 1.1825 | 0.59125 |
| Positives/Total     | 20/20     | 20/20 | 18/20  | 9/20    | 20/20  | 20/20 | 20/20  | 13/20   |
| Mean C <sub>q</sub> | 33.37     | 35.22 | N/A    | N/A     | 31.68  | 32.83 | 33.36  | N/A     |

|                    |                             |      |     |     |                             |      |      |     |
|--------------------|-----------------------------|------|-----|-----|-----------------------------|------|------|-----|
| Standard Deviation | 1.47                        | 1.30 | N/A | N/A | 0.52                        | 0.75 | 0.66 | N/A |
| Limit of Detection | 1.372 copies/20 µl reaction |      |     |     | 0.713 copies/20 µl reaction |      |      |     |

**[00185]** The updated thermal cycling conditions gave better LoD results compared to using the original thermal cycling conditions. LoD of RdRP improved from 5.040 copies/20 µl reaction to 1.372 copies/20 µl reaction; LoD of E improved from 2.375 copies/20 µl reaction to 0.713 copies/20 µl reaction. The updated thermal cycling conditions has thus shown to increase the sensitivity of Acu-Corona® 2.0.

**[00186]** Analytical Specificity and Interference

**[00187]** Limit of Blank

**[00188]** The limit of blank of the assay was tested 40 times by each of two operators in parallel on a single day with water and no other nucleic acid except the provided oligonucleotides (primers and probe) on the Applied Biosystems® 7500 Fast Real-time PCR instrument. Calculation of LoB is based on CLSI guideline EP-17, following the procedure below:

**[00189]** • Due to the inverse nature of Ct values and analyte concentration, sort all B blank sample measurement results from highest to lowest for the target, where B is the total number of results in the dataset: X(1), X(2), ..., X(B).

**[00190]** • Calculate the pth percentile for the distribution of the blank sample results (PcpB) corresponding to the desired α (Type I error) risk probability (e.g., α=0.05 yields p=0.95). For this study, the desired α (Type I error) risk probability shall be 0.05.

**[00191]** • Calculate the rank position corresponding to the PcpB percentile as: Rank Position=0.5+B\*0.95LoB is taken as the value of the measurement result at the rank position calculated above. If the calculated rank position is a non-integer, LoB is calculated by interpolation from measurement results with bracketing rank values. LoB = X (38) +0.5(X (39)-X (38)) = 0.5(X (38) +X (39)).

**[00192]** All the replicates (40 of 40 replicates) had LoB Ct value calls for a target gene be "Undetermined" (data not shown). Hence the LoB test for the gene and performed for that operator shall be the number of qPCR amplification cycles performed.

**[00193]** The limit of blank for both target genes SARS-CoV-2 RdRp gene and E

gene are determined to be the maximum number of run cycles programmed for Acu-Corona™ 2.0: Ct value of 45.00 for SARS-CoV-2 RdRp gene and 45.00 for SARS-CoV-2 E gene.

**[00194]** The limit of blank of the assay was tested 40 times with nuclease-free water and the internal control RNA. The curves and calculated LoB are shown in Table 6.

Table 6: Limit of Blank Cq data for RdRP and E duplexed with the internal control

| Replicate | RdRP              | Internal Control | E                 | Internal Control |
|-----------|-------------------|------------------|-------------------|------------------|
| 1         | No C <sub>q</sub> | 25.28            | No C <sub>q</sub> | 25.37            |
| 2         | No C <sub>q</sub> | 25.19            | No C <sub>q</sub> | 25.57            |
| 3         | No C <sub>q</sub> | 25.26            | No C <sub>q</sub> | 25.48            |
| 4         | No C <sub>q</sub> | 25.46            | No C <sub>q</sub> | 25.51            |
| 5         | No C <sub>q</sub> | 25.36            | No C <sub>q</sub> | 25.92            |
| 6         | No C <sub>q</sub> | 25.32            | No C <sub>q</sub> | 25.55            |
| 7         | No C <sub>q</sub> | 25.18            | No C <sub>q</sub> | 25.38            |
| 8         | No C <sub>q</sub> | 25.18            | No C <sub>q</sub> | 25.68            |
| 9         | No C <sub>q</sub> | 25.25            | No C <sub>q</sub> | 25.44            |
| 10        | No C <sub>q</sub> | 25.37            | No C <sub>q</sub> | 25.62            |
| 11        | No C <sub>q</sub> | 25.38            | No C <sub>q</sub> | 25.40            |
| 12        | No C <sub>q</sub> | 25.28            | No C <sub>q</sub> | 25.25            |
| 13        | No C <sub>q</sub> | 25.19            | No C <sub>q</sub> | 25.36            |
| 14        | No C <sub>q</sub> | 25.35            | No C <sub>q</sub> | 25.35            |
| 15        | No C <sub>q</sub> | 25.18            | No C <sub>q</sub> | 25.44            |
| 16        | No C <sub>q</sub> | 25.50            | No C <sub>q</sub> | 25.32            |
| 17        | No C <sub>q</sub> | 25.20            | No C <sub>q</sub> | 25.29            |
| 18        | No C <sub>q</sub> | 25.15            | No C <sub>q</sub> | 25.25            |
| 19        | No C <sub>q</sub> | 25.16            | No C <sub>q</sub> | 25.26            |
| 20        | No C <sub>q</sub> | 25.23            | No C <sub>q</sub> | 25.43            |
| 21        | No C <sub>q</sub> | 25.25            | No C <sub>q</sub> | 25.43            |
| 22        | No C <sub>q</sub> | 25.16            | No C <sub>q</sub> | 25.22            |
| 23        | No C <sub>q</sub> | 25.19            | No C <sub>q</sub> | 25.30            |
| 24        | No C <sub>q</sub> | 25.25            | No C <sub>q</sub> | 25.35            |
| 25        | No C <sub>q</sub> | 25.29            | No C <sub>q</sub> | 25.75            |
| 26        | No C <sub>q</sub> | 25.32            | No C <sub>q</sub> | 25.32            |
| 27        | No C <sub>q</sub> | 25.14            | No C <sub>q</sub> | 25.23            |
| 28        | No C <sub>q</sub> | 25.14            | No C <sub>q</sub> | 25.38            |
| 29        | No C <sub>q</sub> | 25.21            | No C <sub>q</sub> | 25.51            |
| 30        | No C <sub>q</sub> | 25.18            | No C <sub>q</sub> | 25.42            |
| 31        | No C <sub>q</sub> | 25.24            | No C <sub>q</sub> | 25.36            |
| 32        | No C <sub>q</sub> | 25.20            | No C <sub>q</sub> | 25.41            |
| 33        | No C <sub>q</sub> | 25.21            | No C <sub>q</sub> | 25.41            |
| 34        | No C <sub>q</sub> | 25.26            | No C <sub>q</sub> | 25.33            |
| 35        | No C <sub>q</sub> | 25.29            | No C <sub>q</sub> | 25.55            |
| 36        | No C <sub>q</sub> | 25.28            | No C <sub>q</sub> | 25.56            |
| 37        | No C <sub>q</sub> | 25.44            | No C <sub>q</sub> | 25.38            |
| 38        | No C <sub>q</sub> | 25.27            | No C <sub>q</sub> | 25.46            |
| 39        | No C <sub>q</sub> | 25.32            | No C <sub>q</sub> | 25.49            |
| 40        | No C <sub>q</sub> | 25.31            | No C <sub>q</sub> | 25.69            |

|                           |       |     |       |     |
|---------------------------|-------|-----|-------|-----|
| <b>2<sup>nd</sup> Min</b> | 45.00 | N/A | 45.00 | N/A |
| <b>LoB</b>                | 45.00 | N/A | 45.00 | N/A |

**[00195]** Precision (Repeatability/Reproducibility)

**[00196]** Intermediate precision of the Acu-Corona™ 2.0 assay was evaluated using synthetic dsDNA gBlocks sequences for both SARS-CoV-2 RdRp and E genes. For each gene, three different levels of dilutions of gBlock corresponding to 106 (D2), 104 (D4), and 102 (D6) copies/μl were used.

**[00197]** First, the precision of kits produced at the Research Laboratories (Site A) were tested. For each dilution corresponding to either RdRP or E gene, 33 identical aliquots were each run by two operators (total of 66 replicate reactions per dilution) over a period of two days. The results are summarised in Table 7a, Table 7b, Table 8a, and Table 8b.

**[00198]** Table 6a. Summary analysis of precision variance of Site A kits between two operators across three dilutions for SARS-CoV-2 RdRp gene

| Sample | Mean         | Repeatability SD | Repeatability CV | Within Laboratory SD | Within Laboratory CV |
|--------|--------------|------------------|------------------|----------------------|----------------------|
| D2     | 19.467288480 | 0.110567275      | 0.6%             | 0.238752401          | 1.2%                 |
| D4     | 26.396124782 | 0.098703974      | 0.4%             | 0.213137078          | 0.8%                 |
| D6     | 33.405705555 | 0.271632122      | 0.8%             | 0.297988560          | 0.9%                 |

**[00199]** Table 6b. Precision analysis results of Site A kits between two operators across three dilutions for SARS-CoV-2 RdRp gene

| Sample Pool | Component         | % of Total | SD          | Exact / Satterthwaite<br>95% CI | CV   |
|-------------|-------------------|------------|-------------|---------------------------------|------|
| D2          | Repeatability     | 21.4%      | 0.110567275 | 0.093839523 to<br>0.134608537   | 0.6% |
|             | Between Run       | 78.6%      | 0.211607152 | N/A                             | 1.1% |
|             | Within Operator   | 100.0%     | 0.238752401 | 0.154488671 to<br>0.518877722   | 1.2% |
|             | Between Operator  | 0.0%       | 0.000000000 | N/A                             | 0.0% |
|             | Within Laboratory | 100.0%     | 0.238752401 | 0.159760184 to<br>0.469468337   | 1.2% |
| D4          | Repeatability     | 21.4%      | 0.098703974 | 0.083771024 to<br>0.120165732   | 0.4% |
|             | Between Run       | 65.5%      | 0.172536833 | N/A                             | 0.7% |
|             | Within Operator   | 87.0%      | 0.198774830 | 0.130186234 to<br>0.416137861   | 0.8% |
|             | Between Operator  | 13.0%      | 0.078915413 | N/A                             | 0.3% |
|             | Within Laboratory | 100.0%     | 0.213137078 | 0.135883215 to<br>0.485996545   | 0.8% |
| D6          | Repeatability     | 83.1%      | 0.271632122 | 0.230244636 to<br>0.331299360   | 0.8% |
|             | Between Run       | 16.9%      | 0.122528252 | N/A                             | 0.4% |
|             | Within Operator   | 100.0%     | 0.297988560 | 0.244873451 to<br>0.380740619   | 0.9% |
|             | Between Operator  | 0.0%       | 0.000000000 | N/A                             | 0.0% |
|             | Within Laboratory | 100.0%     | 0.297988560 | 0.250262562 to<br>0.368377856   | 0.9% |

[00200] Table 7a. Summary analysis of precision variance of Site A kits between two operators across three dilutions for SARS-CoV-2 E gene

| Sample | Mean         | Repeatability<br>SD | Repeatability<br>CV | Within<br>Laboratory SD | Within Laboratory<br>CV |
|--------|--------------|---------------------|---------------------|-------------------------|-------------------------|
| D2     | 19.322051453 | 0.030180854         | 0.2%                | 0.097003880             | 0.5%                    |
| D4     | 28.569196175 | 0.046307649         | 0.2%                | 0.287548629             | 1.0%                    |
| D6     | 34.321878086 | 0.380071690         | 1.0%                | 0.663588937             | 1.9%                    |

[00201] Table 7b. Precision analysis results of Site A kits between two operators across three dilutions for SARS-CoV-2 E gene

| Sample Pool | Component         | % of Total | SD          | Exact / Satterthwaite<br>95% CI | CV   |
|-------------|-------------------|------------|-------------|---------------------------------|------|
| D2          | Repeatability     | 9.7%       | 0.030160654 | 0.025597641 to<br>0.036718654   | 0.2% |
|             | Between Run       | 90.3%      | 0.092195899 | N/A                             | 0.5% |
|             | Within Operator   | 100.0%     | 0.097003860 | 0.060119380 to<br>0.244161479   | 0.5% |
|             | Between Operator  | 0.0%       | 0.000000000 | N/A                             | 0.0% |
|             | Within Laboratory | 100.0%     | 0.097003860 | 0.061707726 to<br>0.222818989   | 0.5% |
| D4          | Repeatability     | 3.0%       | 0.046307649 | 0.039301752 to<br>0.056376580   | 0.2% |
|             | Between Run       | 56.3%      | 0.200679101 | N/A                             | 0.8% |
|             | Within Operator   | 58.3%      | 0.205952664 | 0.125575355 to<br>0.551435225   | 0.8% |
|             | Between Operator  | 40.7%      | 0.170779884 | N/A                             | 0.6% |
|             | Within Laboratory | 100.0%     | 0.267548629 | 0.146747493 to<br>1.192703529   | 1.0% |
| D6          | Repeatability     | 29.4%      | 0.360071690 | 0.305596350 to<br>0.438364893   | 1.0% |
|             | Between Run       | 67.0%      | 0.543145853 | N/A                             | 1.6% |
|             | Within Operator   | 96.4%      | 0.651658683 | 0.436512518 to<br>1.277605085   | 1.9% |
|             | Between Operator  | 3.6%       | 0.125264870 | N/A                             | 0.4% |
|             | Within Laboratory | 100.0%     | 0.663588937 | 0.447174409 to<br>1.278501793   | 1.9% |

**[00202]** Intermediate precision of Acu-Corona® 2.0 with updated thermal conditions was evaluated using synthetic dsDNA gBlocks of RdRP and E. Two different dilution levels of gBlocks corresponding to 106 (D2) and 102 (D6) copies per µl were used. 10 replicates were tested for each inter-precision plate per dilution while 20 replicates were tested for the intra-precision plate per dilution. For validating precision, two operators ran a total of three plates (2 inter-precision plates and 1 intra-precision plate). The results of the precision assay are summarised in Tables 9a and 9b.

Table 9a: Intra-precision result table for Acu-Corona® 2.0 on the CFX96 with updated thermal cycling conditions.

| Sample  | Mean      | Repeatability SD | Repeatability CV |
|---------|-----------|------------------|------------------|
| E D2    | 16.303139 | 0.0521847        | 0.3%             |
| E D6    | 29.820319 | 0.1436374        | 0.5%             |
| RdRP D2 | 14.222147 | 0.0753262        | 0.5%             |
| RdRP D6 | 27.1564   | 0.0746161        | 0.3%             |

Table 9b: Inter-precision result table for Acu-Corona® 2.0 on the CFX96 with updated

thermal cycling conditions.

| Sample  | Mean      | Repeatability SD | Repeatability CV | Within Laboratory SD | Within Laboratory CV |
|---------|-----------|------------------|------------------|----------------------|----------------------|
| E D2    | 16.360463 | 0.0618009        | 0.4%             | 0.1137469            | 0.7%                 |
| E D6    | 29.914727 | 0.1224289        | 0.4%             | 0.2039866            | 0.7%                 |
| RdRP D2 | 14.265237 | 0.0617399        | 0.4%             | 0.0823776            | 0.6%                 |
| RdRP D6 | 27.188585 | 0.0640407        | 0.2%             | 0.076109             | 0.3%                 |

**[00203]** Next, the precision of kits produced at the manufacturing site (Site B), the were tested. With the same protocol used for testing the precision of kits produced at site A, 22 identical aliquots were each run by two operators (total of 44 replicate reactions per dilution) within one day. The results are summarised in Table 10a, Table 10b, Table 11a and Table 11b.

**[00204]** Table 10a. Summary analysis of precision variance of Site B kits between two operators across three dilutions for SARS-CoV-2 RdRp gene

| Sample | Mean         | Repeatability SD | Repeatability CV | Within Laboratory SD | Within Laboratory CV |
|--------|--------------|------------------|------------------|----------------------|----------------------|
| D2     | 20.147613742 | 0.095468113      | 0.5%             | 0.135300032          | 0.7%                 |
| D4     | 27.138176701 | 0.221164395      | 0.8%             | 0.366353214          | 1.3%                 |
| D6     | 34.622564749 | 0.399226136      | 1.2%             | 0.711877542          | 2.1%                 |

**[00205]** Table 10b. Precision analysis results of Site B kits between two operators across three dilutions for SARS-CoV-2 RdRp gene

| Sample Pool | Component         | % of Total | SD          | Exact / Satterthwaite<br>95% CI | CV   |
|-------------|-------------------|------------|-------------|---------------------------------|------|
| D2          | Repeatability     | 49.8%      | 0.095468113 | 0.078380552 to<br>0.122151721   | 0.5% |
|             | Between Run       | 48.7%      | 0.094423242 | N/A                             | 0.5% |
|             | Within Operator   | 98.5%      | 0.134275498 | 0.087942466 to<br>0.281111152   | 0.7% |
|             | Between Operator  | 1.5%       | 0.016618946 | N/A                             | 0.1% |
|             | Within Laboratory | 100.0%     | 0.135300032 | 0.092016758 to<br>0.254454497   | 0.7% |
| D4          | Repeatability     | 36.4%      | 0.221164395 | 0.181578820 to<br>0.282980469   | 0.8% |
|             | Between Run       | 37.3%      | 0.223734872 | N/A                             | 0.8% |
|             | Within Operator   | 73.7%      | 0.314596540 | 0.204849767 to<br>0.670411625   | 1.2% |
|             | Between Operator  | 26.3%      | 0.187733040 | N/A                             | 0.7% |
|             | Within Laboratory | 100.0%     | 0.366353214 | 0.221152567 to<br>1.020695153   | 1.3% |
| D6          | Repeatability     | 31.5%      | 0.399226136 | 0.327769805 to<br>0.510810970   | 1.2% |
|             | Between Run       | 68.5%      | 0.589396410 | N/A                             | 1.7% |
|             | Within Operator   | 100.0%     | 0.711877542 | 0.424284950 to<br>2.091117133   | 2.1% |
|             | Between Operator  | 0.0%       | 0.000000000 | N/A                             | 0.0% |
|             | Within Laboratory | 100.0%     | 0.711877542 | 0.463803025 to<br>1.514368869   | 2.1% |

[00206] Table 11a. Summary analysis of precision variance of Site B kits between two operators across three dilutions for SARS-CoV-2 E gene

| Sample | Mean         | Repeatability<br>SD | Repeatability<br>CV | Within<br>Laboratory SD | Within Laboratory<br>CV |
|--------|--------------|---------------------|---------------------|-------------------------|-------------------------|
| D2     | 19.530072732 | 0.042699856         | 0.2%                | 0.388681008             | 2.0%                    |
| D4     | 26.579630765 | 0.044270867         | 0.2%                | 0.546049502             | 2.1%                    |
| D6     | 34.434630134 | 0.443554742         | 1.3%                | 0.607499020             | 1.8%                    |

[00207] Table 11b. Precision analysis results of Site B kits between two operators across three dilutions for SARS-CoV-2 E gene

| Sample Pool | Component         | % of Total | SD          | Exact / Satterthwaite<br>95% CI | CV   |
|-------------|-------------------|------------|-------------|---------------------------------|------|
| D2          | Repeatability     | 1.2%       | 0.042899856 | 0.035221335 to<br>0.054890487   | 0.2% |
|             | Between Run       | 15.5%      | 0.152918761 | N/A                             | 0.8% |
|             | Within Operator   | 16.7%      | 0.158822370 | 0.085137823 to<br>0.816055185   | 0.8% |
|             | Between Operator  | 83.3%      | 0.354751153 | N/A                             | 1.8% |
|             | Within Laboratory | 100.0%     | 0.388681008 | 0.180760571 to<br>7.139046673   | 2.0% |
| D4          | Repeatability     | 0.7%       | 0.044270867 | 0.036346952 to<br>0.058644699   | 0.2% |
|             | Between Run       | 25.6%      | 0.276366962 | N/A                             | 1.0% |
|             | Within Operator   | 26.3%      | 0.279890349 | 0.147169685 to<br>1.638687962   | 1.1% |
|             | Between Operator  | 73.7%      | 0.468861868 | N/A                             | 1.8% |
|             | Within Laboratory | 100.0%     | 0.546049502 | 0.259434008 to<br>7.840361230   | 2.1% |
| D6          | Repeatability     | 53.3%      | 0.443554742 | 0.364164162 to<br>0.567529547   | 1.3% |
|             | Between Run       | 46.7%      | 0.415107517 | N/A                             | 1.2% |
|             | Within Operator   | 100.0%     | 0.607499020 | 0.403606620 to<br>1.219180682   | 1.8% |
|             | Between Operator  | 0.0%       | 0.000000000 | N/A                             | 0.0% |
|             | Within Laboratory | 100.0%     | 0.607499020 | 0.445410284 to<br>0.955141199   | 1.8% |

**[00208]** A combined analysis of the runs conducted using kits produced from Site A and kits produced from Site B was performed to analyse any variation between kits produced at the two sites. The results for this analysis are summarised in Table 12a, Table 12b, Table 13a and Table 13b.

**[00209]** Table 12a. Overall summary analysis of precision variance for SARS-CoV-2 RdRp gene across Site A and Site B, two operators and three dilutions Reagent and Consumables Stability

| Sample | Mean         | Repeatability SD | Repeatability CV | Within Site SD | Within Site CV |
|--------|--------------|------------------|------------------|----------------|----------------|
| D2     | 20.147613742 | 0.095468113      | 0.5%             | 0.135300032    | 0.7%           |
| D4     | 27.138176701 | 0.221164395      | 0.8%             | 0.366353214    | 1.3%           |
| D6     | 34.622564749 | 0.399226136      | 1.2%             | 0.711877542    | 2.1%           |

| Sample | Reproducibility SD | Reproducibility CV |
|--------|--------------------|--------------------|
| D2     | 0.519862005        | 2.6%               |
| D4     | 0.581894960        | 2.2%               |
| D6     | 0.980754352        | 2.9%               |

**[00210]** Table 12b. Overall precision analysis results for SARS-CoV-2 RdRp gene across Site A and Site B, two operators and three dilutions

| Sample Pool | Component        | % of Total | SD          | Exact / Satterthwaite<br>95% CI | CV   |
|-------------|------------------|------------|-------------|---------------------------------|------|
| D2          | Repeatability    | 4.1%       | 0.104789015 | 0.092061550 to<br>0.121632503   | 0.5% |
|             | Between Run      | 12.2%      | 0.181224984 | N/A                             | 0.9% |
|             | Between Operator | 0.0%       | 0.000000000 | N/A                             | 0.0% |
|             | Within Site      | 16.2%      | 0.209339993 | 0.153009417 to<br>0.331321983   | 1.1% |
|             | Between Site     | 83.8%      | 0.475850052 | N/A                             | 2.4% |
|             | Reproducibility  | 100.0%     | 0.519862005 | 0.247015118 to<br>7.456706967   | 2.6% |
| D4          | Repeatability    | 7.5%       | 0.159408157 | 0.140046760 to<br>0.185030971   | 0.6% |
|             | Between Run      | 10.7%      | 0.190510723 | N/A                             | 0.7% |
|             | Between Operator | 5.7%       | 0.138697596 | N/A                             | 0.5% |
|             | Within Site      | 23.9%      | 0.284503637 | 0.197014735 to<br>0.510541229   | 1.1% |
|             | Between Site     | 76.1%      | 0.507601641 | N/A                             | 1.9% |
|             | Reproducibility  | 100.0%     | 0.581894960 | 0.284806168 to<br>6.155708528   | 2.2% |
| D6          | Repeatability    | 12.1%      | 0.341492510 | 0.300015510 to<br>0.396383044   | 1.0% |
|             | Between Run      | 13.5%      | 0.360956253 | N/A                             | 1.1% |
|             | Between Operator | 0.0%       | 0.000000000 | N/A                             | 0.0% |
|             | Within Site      | 25.7%      | 0.496896922 | 0.395660772 to<br>0.668189649   | 1.5% |
|             | Between Site     | 74.3%      | 0.845580493 | N/A                             | 2.5% |
|             | Reproducibility  | 100.0%     | 0.980754352 | 0.486428088 to<br>9.167849646   | 2.9% |

[00211] Table 13a. Overall summary analysis of precision variance for SARS-CoV-2 E gene across Site A and Site B, two operators and three dilutions

| Sample | Mean         | Repeatability<br>SD | Repeatability<br>CV | Within Site SD | Within Site CV |
|--------|--------------|---------------------|---------------------|----------------|----------------|
| D2     | 19.405259965 | 0.035804442         | 0.2%                | 0.253958250    | 1.3%           |
| D4     | 26.573371211 | 0.045503877         | 0.2%                | 0.402762193    | 1.5%           |
| D6     | 34.366978905 | 0.395584753         | 1.2%                | 0.640461077    | 1.9%           |

| Sample | Reproducibility<br>SD | Reproducibility<br>CV |
|--------|-----------------------|-----------------------|
| D2     | 0.253958250           | 1.3%                  |
| D4     | 0.402762193           | 1.5%                  |
| D6     | 0.640461077           | 1.9%                  |

**[00212]** Table 13b. Overall precision analysis results for SARS-CoV-2 E gene across Site A and Site B, two operators and three dilutions.

| Sample Pool | Component        | % of Total | SD          | Exact / Satterthwaite<br>95% CI | CV   |
|-------------|------------------|------------|-------------|---------------------------------|------|
| <b>D2</b>   | Repeatability    | 2.0%       | 0.035804442 | 0.031455706 to<br>0.041559546   | 0.2% |
|             | Between Run      | 20.9%      | 0.115999127 | N/A                             | 0.6% |
|             | Between Operator | 77.1%      | 0.223062855 | N/A                             | 1.1% |
|             | Within Site      | 100.0%     | 0.253958250 | 0.140952241 to<br>1.057440023   | 1.3% |
|             | Between Site     | 0.0%       | 0.000000000 | N/A                             | 0.0% |
|             | Reproducibility  | 100.0%     | 0.253958250 | 0.151946674 to<br>0.733946582   | 1.3% |
| <b>D4</b>   | Repeatability    | 1.3%       | 0.045503877 | 0.039977067 to<br>0.052818041   | 0.2% |
|             | Between Run      | 32.2%      | 0.228711066 | N/A                             | 0.9% |
|             | Between Operator | 66.5%      | 0.328387012 | N/A                             | 1.2% |
|             | Within Site      | 100.0%     | 0.402762193 | 0.229789534 to<br>1.448138974   | 1.5% |
|             | Between Site     | 0.0%       | 0.000000000 | N/A                             | 0.0% |
|             | Reproducibility  | 100.0%     | 0.402762193 | 0.244007803 to<br>1.106008193   | 1.5% |
| <b>D6</b>   | Repeatability    | 36.1%      | 0.395584753 | 0.347537816 to<br>0.459169920   | 1.2% |
|             | Between Run      | 61.9%      | 0.503689482 | N/A                             | 1.5% |
|             | Between Operator | 0.0%       | 0.000000000 | N/A                             | 0.0% |
|             | Within Site      | 100.0%     | 0.640461077 | 0.489708179 to<br>0.925874852   | 1.9% |
|             | Between Site     | 0.0%       | 0.000000000 | N/A                             | 0.0% |
|             | Reproducibility  | 100.0%     | 0.640461077 | 0.498240628 to<br>0.896903167   | 1.9% |

**[00213]** Ct values for the E gene were extremely precise for all three dilution factors as there is a 0.0% increase in coefficient of variation (CV) between within-site CV and reproducibility CV (both fields highlighted in grey in Table 10a). This translates to zero variance between kits produced at the Research Laboratory and those produced at the manufacturing site.

**[00214]** However, a slight difference in Ct values can be observed for the RdRP gene between kits produced at both sites with a maximum of a 1.9% increase in coefficient of variation between within-site CV and reproducibility CV at dilution D2. Nonetheless, reproducibility CV for the RdRP gene remains at less than 3% for all three gBlock dilutions.

**[00215]** Thus, even after taking account variance caused by manufacturing at two different sites, there is no significant difference in SD or CV values for the different sample levels tested (D2, D4, and D6). The analyses show that both sets of precision data from kits manufactured in the Research Laboratory and Manufacturing site are concordant and can produce consistent precision results.

**[00216]** The linearity or dynamic range of the assay was determined by using serial dilutions of synthetic dsDNA template sequence. Each dilution point was tested 3 times by each of two operators in one single day on the Applied Biosystems® 7500 Fast Real-Time PCR instrument. Data are presented in Table 14a and Table 14b.

**[00217]** The results validated the reportable range of 500 to  $5 \times 10^8$  copies of both RdRp target gene and E target gene.

**[00218]** Table 14a. Summary of linear range and amplification efficiencies of SARS-CoV-2 RdRp target gene.

| Copy number (CN)         | Log 10 (CN) | Ct-Op1       | Ct-Op2   |
|--------------------------|-------------|--------------|----------|
| 50                       | 1.7         | Undetermined | Excluded |
|                          | 1.7         | Undetermined | Excluded |
|                          | 1.7         | Undetermined | Excluded |
| 500                      | 2.7         | 32.94957     | 33.43084 |
|                          | 2.7         | 32.9277      | 33.34019 |
|                          | 2.7         | 33.47092     | 33.78271 |
| $5 \times 10^3$          | 3.7         | 29.55187     | 29.94904 |
|                          | 3.7         | 29.59332     | 29.87409 |
|                          | 3.7         | 29.56208     | 29.8094  |
| $5 \times 10^4$          | 4.7         | 26.97786     | 26.78409 |
|                          | 4.7         | 26.92884     | 26.7041  |
|                          | 4.7         | 26.93165     | 26.73586 |
| $5 \times 10^5$          | 5.7         | 23.2374      | 23.75568 |
|                          | 5.7         | 23.22532     | 23.49339 |
|                          | 5.7         | 23.28083     | 23.48644 |
| $5 \times 10^6$          | 6.7         | 19.66572     | 19.52782 |
|                          | 6.7         | 19.59337     | 19.45615 |
|                          | 6.7         | 19.60265     | 19.44931 |
| $5 \times 10^7$          | 7.7         | 15.92466     | 16.23303 |
|                          | 7.7         | 15.99513     | 16.15892 |
|                          | 7.7         | 15.91012     | 16.14321 |
| $5 \times 10^8$          | 8.7         | 12.76156     | 12.55936 |
|                          | 8.7         | 12.71956     | 12.59411 |
|                          | 8.7         | 12.58279     | 12.55016 |
| Slope                    |             | -3.424       | -3.483   |
| Amplification Efficiency |             | 95.93%       | 93.71%   |
| $R^2$                    |             | 0.998        | 0.999    |

**[00219]** Table 14b. Summary of linear range and amplification efficiencies of SARS-CoV-2 E target gene

| Copy number (CN)         | Log 10 (CN) | Ct-Op1   | Ct-Op2   |
|--------------------------|-------------|----------|----------|
| 50                       | 1.7         | 37.52747 | Excluded |
|                          | 1.7         | 37.8864  | Excluded |
|                          | 1.7         | 36.80682 | Excluded |
| 500                      | 2.7         | 31.78831 | 33.57072 |
|                          | 2.7         | 32.56556 | 34.00473 |
|                          | 2.7         | 33.79583 | 34.22849 |
| 5 x 10 <sup>3</sup>      | 3.7         | 29.43358 | 30.22968 |
|                          | 3.7         | 29.4544  | 30.37148 |
|                          | 3.7         | 29.50228 | 30.44879 |
| 5 x 10 <sup>4</sup>      | 4.7         | 26.92549 | 26.66069 |
|                          | 4.7         | 26.92168 | 26.76779 |
|                          | 4.7         | 26.92561 | 26.80698 |
| 5 x 10 <sup>5</sup>      | 5.7         | 23.01505 | 23.26797 |
|                          | 5.7         | 22.66882 | 23.32226 |
|                          | 5.7         | 22.98676 | 23.43543 |
| 5 x 10 <sup>6</sup>      | 6.7         | 19.78973 | 19.2294  |
|                          | 6.7         | 19.57449 | 19.28636 |
|                          | 6.7         | 19.70155 | 19.43150 |
| 5 x 10 <sup>7</sup>      | 7.7         | Excluded | 15.74154 |
|                          | 7.7         | Excluded | 15.83367 |
|                          | 7.7         | Excluded | 15.90156 |
| 5 x 10 <sup>8</sup>      | 8.7         | 11.83092 | 12.46672 |
|                          | 8.7         | 11.8085  | 12.53484 |
|                          | 8.7         | 11.81455 | 12.60564 |
| Slope                    |             | -3.541   | -3.596   |
| Amplification Efficiency |             | 91.61%   | 89.73%   |
| R <sup>2</sup>           |             | 0.994    | 0.999    |

**[00220]** Cross-reactivity with other coronaviruses

**[00221]** Cell culture supernatants containing all endemic human coronaviruses (HCoV)229E, NL63, OC43 and HKU1 as well as MERS-CoV were tested in duplicate in all three assays (table 15). For the non-cultivable HCoV-HKU1, supernatant from human airway culture was used. Viral RNA concentration in all samples was determined by specific real-time RT-PCRs and in vitro-transcribed RNA standards designed for absolute quantification of viral load.

**[00222]** Additional undiluted (but not quantified) cell culture supernatants were tested as summarised in the table 15. These were additionally mixed into negative human sputum samples.<sup>1</sup> None of the tested viruses or virus preparations showed reactivity with any assay.

Table 15 : cross reactivity tests

| Clinical samples with known viruses | Clinical samples <sup>a</sup> | Virus isolates <sup>b</sup> |
|-------------------------------------|-------------------------------|-----------------------------|
| HCoV-HKU1                           | 14                            | 1 <sup>c</sup>              |
| HCoV-OC43                           | 16                            | 2 <sup>d</sup>              |
| HCoV-NL63                           | 14                            | 1 <sup>e</sup>              |
| HCoV-229E                           | 18                            | 2 <sup>f</sup>              |
| MERS-CoV                            | 5                             | 1 <sup>g</sup>              |
| Influenza A(H1N1)pdm09              | 17                            | 1                           |
| Influenza A(H3N2)                   | 16                            | 1                           |
| Influenza A (untyped)               | 11                            | NA                          |
| Influenza A(H5N1)                   | 1                             | 1                           |
| Influenza A(H7N9)                   | 0                             | 1                           |
| Influenza B (Victoria or Yamagata)  | 31                            | 1                           |
| Rhinovirus/enterovirus              | 31                            | NA                          |
| Respiratory syncytial virus (A/B)   | 33                            | NA                          |
| Parainfluenza 1 virus               | 12                            | NA                          |
| Parainfluenza 2 virus               | 11                            | NA                          |
| Parainfluenza 3 virus               | 14                            | NA                          |
| Parainfluenza 4 virus               | 11                            | NA                          |
| Human metapneumovirus               | 16                            | NA                          |
| Adenovirus                          | 13                            | 1                           |
| Human bocavirus                     | 6                             | NA                          |
| Legionella spp.                     | 3                             | NA                          |
| Mycoplasma spp.                     | 4                             | NA                          |
| Total clinical samples              | 297                           | NA                          |

**[00223]** When intermediate precision was assessed at the Cq level for both genes:

Repeatability SD < 0.2 cycles

Repeatability CV ≤ 0.4%

Within-laboratory SD < 0.3 cycles

Within-laboratory CV  $\leq 0.7\%$

**[00224]** Cross-reactivity with other coronaviruses

**[00225]** In silico analysis of primer and probe cross-reactivity for all three gene targets were performed for all organisms listed in Table 16.

**[00226]** RdRP gene: F-primer, R-primer, probe

**[00227]** E gene: F-primer, R-primer, probe

**[00228]** MS2 target region: F-primer, R-primer, probe

**[00229]** In silico analysis of all three primer-probe pairs for cross-reactivity were performed using BLASTn analysis queries against public domain nucleotide sequences. The database search parameters were as follows:

**[00230]** Database: Nucleotide collection (nt) consisting of GenBank + EMBL + DDBJ + PDB + RefSeq sequences, but excludes EST, STS, GSS, WGS, TSA, patent sequences as well as phase 0, 1, and 2 HTGS sequences and sequences longer than 100Mb. The database is non-redundant; identical sequences have been merged into one entry, while preserving the accession, GI, title, and taxonomy information for each entry.

**[00231]** No models (XM/XP) or uncultured/environmental sample sequences were excluded.

**[00232]** The program was optimised for highly similar sequences (i.e. megablast BLAST algorithm was selected).

**[00233]** Automatic adjusting of parameters for short input sequences was selected.

**[00234]** Expect threshold was set at 1,000.

**[0001]** Match and Mismatch scores were set at 1 and -3, respectively.

**[00235]** The penalty to create and extend a gap in an alignment was 5 and 2, respectively.

**[00236]** Conclusion: The RdRP assay has 81~100% homology with SARS-coronavirus (Tax id 694009), MERS-coronavirus (1335626), Human coronavirus HKU1 (290028), Human coronavirus 229E (11137), Human coronavirus OC43 (31631), and

Human coronavirus NL63 (277944), but <80% homology with all other organisms listed in Table 16.

**[00237]** The E assay has 82~100% homology with Human coronavirus (694009) and Influenza A (11320), but <80% homology with all other organisms listed in Table 16.

**[00238]** Refer to Table 16 for cross-reactivity in silico study results and Table 10 for accession numbers of organism strains with more than 80% homology with RdRP gene and E gene primers and probes.

Table 16: Organisms assessed in silico for potential cross-reactivity; degrees of homology between the primer/probe against full length genome of the pathogens are presented as percentages

| Organism (Taxid)                                                    | RdRP Gene |          |       | E Gene   |          |       | MS2 Target Region |          |       |
|---------------------------------------------------------------------|-----------|----------|-------|----------|----------|-------|-------------------|----------|-------|
|                                                                     | F Primer  | R Primer | Probe | F Primer | R Primer | Probe | F Primer          | R Primer | Probe |
| <b>High priority pathogens from the same genetic family</b>         |           |          |       |          |          |       |                   |          |       |
| <i>SARS-coronavirus</i> (694009)                                    | 100%      | 96%      | 100%  | 100%     | 95%      | 100%  | <80%              | <80%     | <80%  |
| <i>MERS-coronavirus</i> (1335626)                                   | <80%      | 88%      | <80%  | <80%     | <80%     | <80%  | <80%              | <80%     | <80%  |
| <i>Human coronavirus 229E</i> (11137)                               | <80%      | 81%      | <80%  | <80%     | <80%     | <80%  | <80%              | <80%     | <80%  |
| <i>Human coronavirus OC43</i> (31631)                               | 82%       | <80%     | <80%  | <80%     | <80%     | <80%  | <80%              | <80%     | <80%  |
| <i>Human coronavirus HKU1</i> (290028)                              | 91%       | <80%     | <80%  | <80%     | <80%     | <80%  | <80%              | <80%     | <80%  |
| <i>Human coronavirus NL63</i> (277944)                              | <80%      | 85%      | <80%  | <80%     | <80%     | <80%  | <80%              | <80%     | <80%  |
| <b>High priority organisms likely to be in the circulating area</b> |           |          |       |          |          |       |                   |          |       |
| <i>Mycoplasma pneumoniae</i> (2104)                                 | <80%      | <80%     | <80%  | <80%     | <80%     | <80%  | <80%              | <80%     | <80%  |
| <i>Adenovirus 4</i> (28280)                                         | <80%      | <80%     | <80%  | <80%     | <80%     | <80%  | <80%              | <80%     | <80%  |
| <i>Adenovirus 7</i> (10519)                                         | <80%      | <80%     | <80%  | <80%     | <80%     | <80%  | <80%              | <80%     | <80%  |
| <i>Human Metapneumovirus (hMPV)</i> (162145)                        | <80%      | <80%     | <80%  | <80%     | <80%     | <80%  | <80%              | <80%     | <80%  |
| <i>Parainfluenza virus 1</i> (12730)                                | <80%      | <80%     | <80%  | <80%     | <80%     | <80%  | <80%              | <80%     | <80%  |
| <i>Parainfluenza virus 2</i> (1979160)                              | <80%      | <80%     | <80%  | <80%     | <80%     | <80%  | <80%              | <80%     | <80%  |
| <i>Parainfluenza virus 3</i> (11216)                                | <80%      | <80%     | <80%  | <80%     | <80%     | <80%  | <80%              | <80%     | <80%  |
| <i>Parainfluenza virus 4a</i> (11224)                               | <80%      | <80%     | <80%  | <80%     | <80%     | <80%  | <80%              | <80%     | <80%  |
| <i>Parainfluenza virus 4b</i> (11226)                               | <80%      | <80%     | <80%  | <80%     | <80%     | <80%  | <80%              | <80%     | <80%  |
| <i>Influenza A</i> (11320)                                          | <80%      | <80%     | <80%  | <80%     | 82%      | <80%  | <80%              | <80%     | <80%  |
| <i>Influenza B</i> (11520)                                          | <80%      | <80%     | <80%  | <80%     | <80%     | <80%  | <80%              | <80%     | <80%  |
| <i>Enterovirus EV68</i> (42789)                                     | <80%      | <80%     | <80%  | <80%     | <80%     | <80%  | <80%              | <80%     | <80%  |
| <i>Respiratory syncytial virus</i> (11250)                          | <80%      | <80%     | <80%  | <80%     | <80%     | <80%  | <80%              | <80%     | <80%  |
| <i>Rhinovirus</i> (12059)                                           | <80%      | <80%     | <80%  | <80%     | <80%     | <80%  | <80%              | <80%     | <80%  |
| <i>Chlamydia pneumoniae</i> (83558)                                 | <80%      | <80%     | <80%  | <80%     | <80%     | <80%  | <80%              | <80%     | <80%  |
| <i>Haemophilus influenzae</i> (727)                                 | <80%      | <80%     | <80%  | <80%     | <80%     | <80%  | <80%              | <80%     | <80%  |
| <i>Legionella pneumophila</i> (446)                                 | <80%      | <80%     | <80%  | <80%     | <80%     | <80%  | <80%              | <80%     | <80%  |

|                                                   |      |      |      |      |      |      |      |      |      |
|---------------------------------------------------|------|------|------|------|------|------|------|------|------|
| <i>Mycobacterium tuberculosis</i> complex (77643) | <80% | <80% | <80% | <80% | <80% | <80% | <80% | <80% | <80% |
| <i>Streptococcus pneumoniae</i> (1313)            | <80% | <80% | <80% | <80% | <80% | <80% | <80% | <80% | <80% |
| <i>Streptococcus pyogenes</i> (1314)              | <80% | <80% | <80% | <80% | <80% | <80% | <80% | <80% | <80% |
| <i>Bordetella pertussis</i> (520)                 | <80% | <80% | <80% | <80% | <80% | <80% | <80% | <80% | <80% |
| <i>Pneumocystis jirovecii</i> (PJP) (42068)       | <80% | <80% | <80% | <80% | <80% | <80% | <80% | <80% | <80% |
| <i>Candida albicans</i> (5476)                    | <80% | <80% | <80% | <80% | <80% | <80% | <80% | <80% | <80% |
| <i>Pseudomonas aeruginosa</i> (287)               | <80% | <80% | <80% | <80% | <80% | <80% | <80% | <80% | <80% |
| <i>Staphylococcus epidermidis</i> (1282)          | <80% | <80% | <80% | <80% | <80% | <80% | <80% | <80% | <80% |
| <i>Streptococcus salivarius</i> (1304)            | <80% | <80% | <80% | <80% | <80% | <80% | <80% | <80% | <80% |

Table 17: Accession numbers for organisms with &gt; 80% homology with primers and probes

| Primer / Probe            | Organism (Taxid)                       | Accession number | Homology |
|---------------------------|----------------------------------------|------------------|----------|
| <b>RdRP Gene F Primer</b> | <i>SARS-coronavirus</i> (694009)       | MT669322.1       | 100%     |
|                           | <i>Human coronavirus HKU1</i> (290028) | KX898512.1       | 91%      |
|                           | <i>Human coronavirus OC43</i> (31631)  | KF923924.1       | 82%      |
| <b>RdRP Gene R Primer</b> | <i>SARS-coronavirus</i> (694009)       | AY465927.1       | 96%      |
|                           | <i>MERS-coronavirus</i> (1335626)      | MN312847.1       | 88%      |
|                           | <i>Human coronavirus 229E</i> (11137)  | KT253272.1       | 81%      |
|                           | <i>Human coronavirus NL63</i> (277944) | MN306040.1       | 85%      |
| <b>RdRP Gene Probe</b>    | <i>SARS-coronavirus</i> (694009)       | MN996532.1       | 100%     |
| <b>E Gene F Primer</b>    | <i>SARS-coronavirus</i> (694009)       | MT308984.1       | 100%     |
| <b>E Gene R Primer</b>    | <i>SARS-coronavirus</i> (694009)       | MN312859.1       | 95%      |
|                           | <i>Influenza A</i> (11320)             | KY614927.1       | 82%      |
| <b>E Gene Probe</b>       | <i>SARS-coronavirus</i> (694009)       | MT308984.1       | 100%     |

**[00239]** Primer probe design

**[00240]** This real-time RT-PCR assay targeting the RdRP and E genes of COVID-19 was designed based on the first publicly available sequence in Genbank (Accession number: MN908947). The downloaded sequence and those for SARS coronavirus, bat SARS-like coronaviruses, and other representative coronaviruses were edited and aligned. The sequence region of RdRP and E gene which is highly conserved was used for primer and probe design. The primer and probe sequences for the **Acu-Corona™ 2.0 assay** are:

**[00241]** COVID-19 RdRp Gene

**[00242]** Primer (F) Sequence: GTGARATGGTCATGTGTGGCGG

**[00243]** Primer (R) Sequence: CARATGTTAAASACACTATTAGCATA

- [00244] Probe Sequence: /56-  
FAM/CAGGTGGAACCTCATCAGGAGATGC/3BBQ650/
- [00245] COVID-19 E Gene
- [00246] Primer (F) Sequence: ACAGGTACGTTAATAGTTAATAGCGT
- [00247] Primer (R) Sequence: ATATTGCAGCAGTACGCACACA
- [00248] Probe Sequence: /56-  
FAM/ACACTAGCCATCCTTACTGCGCTTCG/3BBQ650/
- [00249] Internal Control Target
- [00250] Primer (F) Sequence: TGGCACTACCCCTCTCCGTATTCAC
- [00251] Primer (R) Sequence: GTACGGGCGACCCACGATGAC
- [00252] Probe Sequence: /56-  
JOEN/CACATCGATAGATCAAGGTGCCTACAAGC/3BHQ\_1/
- [00253] In-silico analysis of the sequence
- [00254] The designed primer and probe sequences of Acu-Corona™ 2.0 assay were aligned with publicly available nucleic acid sequences for COVID-19 in GenBank as of 23 Mar 2020 to demonstrate the predicted inclusivity of Acu-Corona™ 2.0. Two GenBank full genome sequences were manually selected, in total 93 sequences were included in the analysis. For primer and probe sequences of E gene, forward primer and probe sequences of RdRP gene, all alignments show 100% identity of the Acu-Corona™ 2.0 sequences to available COVID-19 genomic sequences. The reverse primer of RdRP gene showed one mismatch with the COVID-19 genome at position 12 due to limited sequence information when the primer was designed. Experimental validation showed this would not affect the assay. Combining primers and probe, there is no prediction of potential false positive RT-PCR results.
- [00255] Acu-Corona™ 2.0 has been validated for use with the following equipment and consumables however it may be replaced by other equivalent equipment.
- 1a. Applied Biosystems® 7500 Fast Real-Time PCR System, Cat No. 5 4351107

1b. Applied Biosystems® 7500 Software v2.3 or higher

**[00256]** Please use the RNA extraction and purification kit selected by your laboratory as per laboratory quality standards and requirements.

**[00257]** The following general laboratory equipment is required to perform 10 Acu-Corona™ 2.0.

**[00258]** All laboratory equipment should be installed, calibrated, operated, and maintained according to the manufacturer's recommendations and according to the laboratory's quality standards prior to performing the test.

| Equipment                                                                   | Recommended Equipment                                         |
|-----------------------------------------------------------------------------|---------------------------------------------------------------|
| Adjustable micropipettes for: 0.5µL to 10µL, 10µL to 100µL, 100µL to 1000µL | Rainin Cat No 17014388, 17014384 and 17014382 (or equivalent) |
| Tube rack for 0.2mL thin walled PCR tubes                                   | Neptune, Cat No 3423.S.X (or equivalent)                      |
| Bench top centrifuge with rotor for 0.2mL tubes                             | Hoefer, Cat No. CF 1000-230V (or equivalent)                  |
| Bench top centrifuge with rotor for 1.5/0.2mL tubes                         | Eppendorf, Cat No. 022620304 (or equivalent)                  |
| Repeater micropipette for 10 µL volume repeats                              | Rainin, Cat No. 17014487 (or equivalent)                      |
| Plate centrifuge for 1,000 RCF centrifugation                               | Eppendorf, Cat No. 5427000011 (or equivalent)                 |

**[00259]** The following general laboratory consumables and reagents are required to perform Acu-Corona™ 2.0 and are not provided in the Acu-Corona™ 2.0 kits. **Equivalent or similar products may be used, according to the laboratory's quality standards.**

| Materials                                   | Recommended Materials                                |
|---------------------------------------------|------------------------------------------------------|
| RNase surface decontaminant                 | Molecular BioProducts™, Cat No. 7000 (or equivalent) |
| 0.2 mL thin wall PCR microtubes, RNase-free | Neptune®, Cat No. 3423.S.X. (or equivalent)          |
| 0.2 mL microtubes, RNase-free               | Axygen®, Cat No. MCT-200-C-S (or equivalent)         |

|                                               |                                                 |
|-----------------------------------------------|-------------------------------------------------|
| Tips for repeat pipettor (10µL), RNase-Free   | Rainin, Cat No. 30389226                        |
| Pipette tips with aerosol barrier, RNase-Free | Rainin Cat No. 17007957, 17007961, and 17007954 |
| Dry, lint-free laboratory wipe                | Kim Tech Sciences Cat No. 34155                 |

**[00260]** Test Workflow

**[00261]** Acu-Corona™ 2.0 begins with patient RNA samples extracted and purified through an RNA extraction system.

**[00262]** The RNA input sample should be of high quality as per the laboratory's standards.

**[00263]** Use the RNA extraction and purification kit selected by the laboratory as per laboratory quality standards and requirements. Refer to the manufacturer's instructions of these RNA extraction and purification systems prior to use.

**[00264]** Prepare 200 µL neat patient sample ready for RNA extraction.

**[00265]** Fully thaw Acu-Corona® 2.0 Internal Control on ice.

**[00266]** Aliquot Acu-Corona® 2.0 Internal Control into the patient sample with a dilution factor of 1:100 and mix thoroughly by pipetting.

**[00267]** Wipe down the work bench and bench instruments and ice container with 70% ethanol and a nuclease decontaminant.

**[00268]** Thaw Acu-Corona® 2.0 4x Reaction Mix and Positive Control on ice.

**[00269]** Thaw Acu-Corona® 2.0 Nuclease-free Water (on ice or without ice).

**[00270]** Vortex Acu-Corona® 2.0 4x Reaction Mix for 20-25 sec at low speed and then pulse spin for 3 sec.

**[00271]** Vortex Acu-Corona® 2.0 Nuclease-Free Water and Positive Control for 3 sec at high speed and then pulse spin for 3 sec.

**[00272]** Prepare the master mix for RT-qPCR according to Table 18.

**[00273]** Vortex the prepared master mix at low speed for 3 sec and pulse spin for 3 sec.

Table 18: Acu-Corona® 2.0 Master Mix formulation, where S is the total number of patient specimens to be run.

| Reagent                             | Vol. for each well | Total Master Mix Vol;<br>For <u>1-42</u> patient samples per run | Total Master Mix Vol;<br>For <u>43-46</u> patient samples per run |
|-------------------------------------|--------------------|------------------------------------------------------------------|-------------------------------------------------------------------|
| Acu-Corona® 2.0 4x Reaction Mix     | 5 µL               | $(5.2 \times 2S + 20) \mu\text{L}$                               | 495 µL                                                            |
| Acu-Corona® 2.0 Nuclease-Free Water | 5 µL               | $(5.2 \times 2S + 20) \mu\text{L}$                               | 495 µL                                                            |
| Final volume                        | 10 µL              | $(10.4 \times 2S + 40) \mu\text{L}$                              | 990 µL                                                            |

**[00274]** Plate Loading

**[00275]** Retrieve one Acu-Corona® 2.0 plate; remove the adhesive foil gently.

**[00276]** Dispense 10 µL of the prepared Master Mix into every well used, including NPC and PPC wells. Carefully dispense the master mix onto the side wall of the wells; DO NOT touch the bottom of the well.

**[00277]** Aliquot 10 µL of patient RNA eluate directly into the bottom of the sample wells according to Figure 1. DO NOT touch the inner walls of the wells.

**[00278]** Add 10 µL of Acu-Corona® 2.0 Nuclease-Free Water directly into the bottom of NPC1 and NPC2 wells. Change a pipette tip after each well and do not touch the inner walls of the wells.

**[00279]** Add 5 µL of Acu-Corona® 2.0 Positive PCR Control directly into the bottom of PPC1 and PP2 wells. Change a pipette tip after each well and do not touch the inner walls of the wells.

**[00280]** Add 5 µL of Acu-Corona® 2.0 Nuclease-Free Water directly into the bottom of PPC1 and PP2 wells.

**[00281]** Tap the plate gently on the benchtop to settle the liquid drops in the wells.

**[00282]** Seal the plate with the provided plate sealing film. Press down firmly along

the borders of all wells to ensure all individual wells are properly sealed.

**[00283]** Vortex each plate at high speed for 15 to 20 seconds.

**[00284]** Centrifuge each plate at 1,000 RCF (1,000 × g) for 3 minutes.

**[00285]** Run the RT-qPCR reaction using a PCR equipment validated in the laboratory with the programmed protocol pre-set in Table 19.

Table 19: PCR System Protocol Set-Up Parameters

| Program               | No. of Cycles | Target | Hold (mm:ss) |
|-----------------------|---------------|--------|--------------|
| Reverse Transcription | 1             | 50.0°C | 05:00        |
|                       |               | 95.0°C | 00:20        |
| qPCR Amplification    | 45            | 95.0°C | 00:05        |
|                       |               | 58.0°C | 00:30        |

**[00286]** Alternative Acu-Corona™ 2.0 One-Step Reverse Transcription and Quantitative PCR Amplification & Detection Acu-Corona™ 2.0 also targets two RNA sequences unique to the SARS-CoV-2 virus, in addition to one RNA sequence unique to the genetic material in the provided internal control. One-step qRT-PCR transcribes these RNA regions to cDNA, which is then simultaneously amplified and detected by the ABI 7500 Fast PCR equipment.

**[00287]** In this alternative PCR amplicons are detected by measuring fluorescence intensities. Three different probes each contain a fluorescence-quencher pair; the latter of which subdues the former under normal conditions. When the probe binds to cDNA amplicons, Taq polymerase in the 4x Reaction Mix excises the fluorescence from the probe, allowing it to dissociate away from the quencher, thereby emitting fluorescence. These fluorescent 15 signals are detected and their intensities are measured in real-time during qPCR. Acu-Corona™ 2.0 specifically targets two SARS-CoV-2 RNA regions. A positive result indicates SARS-CoV-2 infection.

**[00288]** 1. Retrieve one Acu-Corona™ 2.0 96-Well Array Plate; remove the adhesive foil gently. 20

**[00289]** 2. Dispense 15 µL of the Master Mix into every well used, including the four NPC and PPC wells. Carefully dispense the master mix onto the side wall of the wells; DO

NOT touch the bottom of the well.

**[00290]**      3. Aliquot 5 µL of patient RNA elute directly into the bottom of the sample wells according to Figure 1. DO NOT touch the inner walls of the wells. Add to wells indicated by or (refer to Figure 1).

**[00291]**      4. Add 5 µL of Acu-Corona™ 2.0 Nuclease-Free Water directly into the bottom of the negative control wells according to Figure 1. DO NOT touch the inner walls of the wells. Add to two wells indicated by Negative PCR Control.

**[00292]**      5. Add or 5 µL of Acu-Corona™ Positive PCR Control directly into the bottom of the positive control wells according to Figure 1. DO NOT touch the inner walls of the wells. Add to two wells indicated by Positive PCR Control

**[00293]**      6. Tap the plate gently on the table to settle the liquid drops in the wells.

**[00294]**      7. Seal the plate with an Acu-Corona™ 2.0 Plate Sealing Film. Press down firmly to ensure all individual wells are properly sealed.

**[00295]**      8. Vortex each plate at high speed for 15 to 20 seconds.

**[00296]**      9. Centrifuge each plate at 1,000 RCF (1,000 g) for 3 minutes. Note: After centrifugation, make sure there are no bubbles within the wells. If bubbles are present, flick the bubbles away and centrifuge the plate again at the same setting.

**[00297]**      10. Run the qRT-PCR reaction using an Applied Biosystems® 7500 Fast Real-Time PCR System (Section 9.5) with the programmed protocol pre-set to the respective specifications.

**[00298]**      11. Discard the Acu-Corona™ 2.0 96-Well Array Plate after the run is 20 complete. DO NOT attempt to remove the plate sealing film upon completion of the run.  
Figure 1: Loading layout for Acu-Corona™ 2.0 96-Well Array Plate PPC NPC

**[00299]**      While Acu-Corona™ 2.0 Kit A components require standard -20°C cold-chain shipping and storage conditions, primers and probes—normally shipped via cold-chain—are specially pre-printed within the Acu-Corona™ 2.0 plates. This printed and dried form allows long-term ambient temperature storage (i.e. at least six months in ambient storage conditions), further ensuring that these reagents critical for PCR reactions are not compromised by temperature during any part of the kit delivery.

**[00300]** Prevention of Contamination and Errors

**[00301]** By minimising the number of reagent handling steps needed in reaction set-up in the testing laboratory, sample handling and pipetting steps are reduced, lowering the critical risks of sample degradation, cross-contamination, and critically, human error.

**[00302]** Convenience

**[00303]** Acu-Corona™ 2.0 eliminates Manual pipetting of excessive PCR reagents create the potential for technical errors, poor inconsistencies by virtue of their labour-intensive nature and thus poor result reproducibility. Using reagents pre-printed and prepared in the 96-well reaction plates offers ease-of-use and minimises hands-on time, which are essential for improved laboratory through-put as well as yielding consistent and timely results.

**[00304]** Robustness

**[00305]** The kit design reduces the number of freeze-thaw cycles that reagents are subject to; critical reagents in Acu-Corona™ 2.0 Kit A (requiring **-20°C storage conditions**) are aliquoted in small volumes in multiple vials to reduce degradation due to freeze-thaw cycles.

**[00306]** It should be further appreciated by the person skilled in the art that variations and combinations of features described above, not being alternatives or substitutes, may be combined to form yet further embodiments falling within the intended scope of the invention.

**[00307]** As would be understood by a person skilled in the art, each embodiment, may be used in combination with other embodiment or several embodiments.