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(54) Title: COMPOSITIONS, KITS AND METHODS FOR DETECTION OF VIRAL SEQUENCES

	Strain	Complete genome (%)	Gene region (%)												
			1ab	1a	1b	S	3	E	M	7	8	10b	N	13	14
Nucleotide sequences	Bat-SL-CoVZC45	87.6	88.9	90.7	86.0	75.2	87.8	98.7	93.4	95.2	88.8	88.5	91.1	89.1	96.7
	Bat-SL-CoVZXC21	87.5	88.7	90.3	86.1	74.7	88.9	98.7	93.4	95.2	89.1	88.5	91.2	89.5	96.7
	SARS-CoVGZ02	79.0	79.5	75.4	86.3	72.7	75.6	93.5	85.1	74.5	82.1	..	88.1	..	..
Amino acid sequences	Bat-SL-CoVZC45	..	95.6	95.6	95.8	80.2	90.9	100.0	98.6	93.4	87.6	94.2	94.3	73.2	92.9
	Bat-SL-CoVZXC21	..	95.2	95.1	95.5	79.6	92.0	100.0	98.6	93.4	88.4	94.2	94.3	73.2	92.9
	SARS-CoVGZ02	..	86.2	80.5	95.6	76.2	73.1	94.7	90.1	88.9	85.2	..	90.3	..	..

FIG. 1A

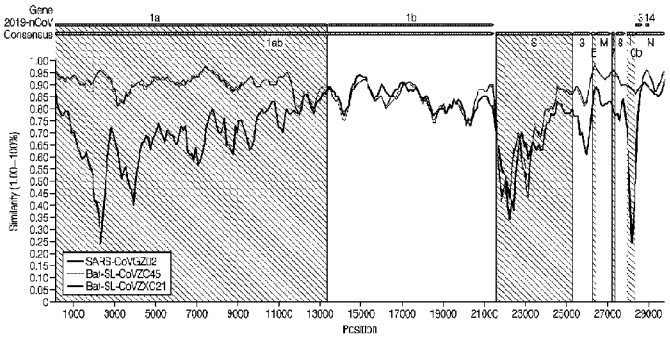


FIG. 1B

(57) Abstract: Compositions, assays, methods, diagnostic methods, kits, and diagnostic kits are disclosed for the specific and differential detection of SARS-CoV-2 and/or other viruses from samples, including veterinary samples, clinical samples, food samples, forensic sample, environmental samples (e.g., obtained from soil, garbage, sewage, air, water, food processing and manufacturing surfaces, or likewise), or biological sample obtained from a human or non-human animal.

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[Continued on next page]

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COMPOSITIONS, KITS, AND METHODS FOR DETECTION OF VIRAL SEQUENCES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to and the benefit of United States Provisional Patent Application Serial No. 63/199,570, filed January 8, 2021 and titled "COMPOSITIONS, KITS AND METHODS FOR DETECTION OF VIRAL SEQUENCES"; United States Provisional Patent Application Serial No. 63/199,076, filed December 4, 2020 and titled "COMPOSITIONS, KITS AND METHODS FOR DETECTION OF VIRAL SEQUENCES"; United States Provisional Patent Application Serial No. 63/198,421, filed October 16, 2020 and titled "COMPOSITIONS, KITS AND METHODS FOR DETECTION OF VIRAL SEQUENCES"; United States Provisional Patent Application Serial No. 63/198,134, filed September 30, 2020 and titled "COMPOSITIONS, KITS AND METHODS FOR DETECTION OF VIRAL SEQUENCES"; United States Provisional Patent Application Serial No. 62/706,081, filed July 30, 2020 and titled "COMPOSITIONS, KITS AND METHODS FOR DETECTION OF VIRAL SEQUENCES"; United States Provisional Patent Application Serial No. 63/052,385, filed July 15, 2020 and titled "COMPOSITIONS, KITS AND METHODS FOR DETECTION OF VIRAL SEQUENCES"; United States Provisional Patent Application Serial No. 63/044,160, filed June 25, 2020 and titled "COMPOSITIONS, KITS AND METHODS FOR DETECTION OF VIRAL SEQUENCES"; United States Provisional Patent Application Serial No. 62/981,938, filed February 26, 2020 and titled "COMPOSITIONS, KITS AND METHODS FOR DETECTION OF VIRAL SEQUENCES"; and United States Provisional Patent Application Serial No. 62/978,274, filed February 18, 2020 and titled "COMPOSITIONS, KITS AND METHODS FOR DETECTION OF VIRAL SEQUENCES." Each of the foregoing applications are incorporated herein by this reference in their entirety.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on January 15, 2021, is named LT01529PCT_SL.txt and is 665,718 bytes in size.

FIELD

[0003] The present teachings relate to compositions, methods, systems and kits for specific detection, diagnosis and differentiation of viruses involved in infectious diseases. Differential

detection of specific viral agents allows accurate diagnosis so that appropriate treatment and infection control measures can be provided in a timely manner.

BACKGROUND

[0004] Infectious diseases are caused by pathogenic microbes or infectious agents (e.g., viruses). Early and accurate diagnosis of infectious disease is important for several reasons. For example, proper diagnosis can lead to earlier, more effective treatment which improves outcomes for the infected individual. On the other hand, individuals who are undiagnosed or misdiagnosed may unknowingly transmit diseases to others. Accurate diagnoses also help ensure proper treatments are applied, particularly with respect to certain disease categories with multiple pathogenic causes and similar symptom profiles, such as respiratory diseases.

[0005] One example of a problematic virus associated with infectious diseases are coronaviruses. Coronaviruses are a family of viruses having a positive-sense single stranded RNA genome of about 30 kilobases in length. Human coronaviruses were first identified in the mid 1960's as being one of the many etiologic agents of the common cold. People around the world commonly get infected with human coronavirus strains 229E (an alpha coronavirus), NL63 (an alpha coronavirus), OC43 (a beta coronavirus), and HKU1 (a beta coronavirus). These infections present with mild clinical symptoms and are associated with an extremely low mortality rate.

[0006] Some coronaviruses infect non-human animals where they can evolve and undergo zoonosis, expanding their tropism to humans. Such crossover events have proven devastating in years past. For example, the Middle East Respiratory Syndrome (MERS) was caused by MERS-CoV, a beta coronavirus that crossed over from dromedary camels to humans. MERS-CoV was associated with a high mortality rate of approximately 35%, but its low transmissibility rate helped to limit its spread and potential for devastation. As another example, Severe Acute Respiratory Syndrome (SARS), which was caused by SARS-CoV, another beta coronavirus, was believed to have been transmitted from bats to civet cats who then transmitted the virus to humans. Although not as deadly as MERS-CoV, SARS-CoV was nevertheless associated with a moderately high mortality rate of approximately 9.6%. Likely due, at least in part, to the lifecycle of SARS-CoV within humans, the spread of this virus was limited mostly to southeast Asian countries. Human infected with SARS-CoV often became symptomatic prior to shedding infectious virions, making quarantining a particularly useful tool for limiting exposure and spread of the infection.

[0007] More recently, a new variant beta coronavirus, SARS-CoV-2 (also known as 2019-nCoV), has emerged, potentially from a crossover event between pangolins and humans in Wuhan, China. While the epidemiological data are incomplete, reports so far indicate that over 85 million

people worldwide are believed to have already been infected by SARS-CoV-2. However, unlike MERS-CoV and SARS-CoV before it, SARS-CoV-2 appears to be significantly less lethal on average with a mortality rate of about 2.3%. Due to its increased transmissibility, the seemingly small percentage of deaths associated with SARS-CoV-2 belies its worldwide impact, having caused an estimated 1.9 million deaths in the worldwide pandemic at the time of this filing, and currently continuing to grow. The raw number of humans impacted by SARS-CoV-2 dwarfs the total number of deaths caused by MERS-CoV and SARS-CoV combined—reportedly around 1,600.

[0008] Given the present and continuing emergence of new coronavirus strains, there is an urgent need to develop methods for the rapid detection and characterization of existing and novel coronavirus strains so that appropriate treatment and infection control measures can be properly instituted in a timely manner. Problematically, many of the SARS-CoV-2 detection assays are non-specific with respect to detecting and differentiating SARS-CoV-2 from other respiratory pathogens, particularly other coronaviruses, which has led to a lack of patient confidence in the diagnostic potential of current SARS-CoV-2 detection assays. Further, because individuals infected with SARS-CoV-2 often experience symptoms similar to those infected with Influenza Types A or B (Flu A or Flu B) and/or Respiratory Syncytial Virus (RSV), there is an additional need to be able to simultaneously test for each of these respiratory viruses in order to provide an accurate diagnosis before seeking/providing treatment and/or confining the individual to a quarantined area under the potentially mistaken belief that they are infected with SARS-CoV-2. Each misidentified or misdiagnosed instance of SARS-CoV-2 infection further convolutes the epidemiological data and prevents the implementation of appropriate, informed solutions.

[0009] Accordingly, there are a number of disadvantages with current methods, systems, compositions, and kits for detecting existing and novel coronavirus strains among other common respiratory tract viral pathogens, which can be addressed, and the methods, systems, compositions, and kits of the present disclosure address and overcome at least some of the foregoing problems in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] In order to describe the manner in which the above recited and other advantages and features of the disclosure can be obtained, a more particular description of the disclosure briefly described above will be rendered by reference to specific embodiments thereof, which are illustrated in the appended drawings. It is appreciated that these drawings depict only typical embodiments of the disclosure and are not therefore to be considered to be limiting of its scope.

[0011] The disclosure will be described and explained with additional specificity and detail through the use of the accompanying drawings in which:

[0012] FIG. 1A illustrates the sequence identity between the consensus SARS-CoV-2 sequence and three closely related coronaviruses, namely, Bat-SL-CoVZC45, Bat-SL-CoVZXC21, and SARS-CoVGZ02, across the complete genome and across specific gene regions within the coronavirus genome.

[0013] FIG. 1B illustrates the tabular information of FIG. 1A in graphical form with the x-axis being the base pair position within the viral genome and the y-axis being the percent similarity of each related virus to the corresponding SARS-CoV-2 consensus sequence.

[0014] FIG. 2A illustrates the amplification plot of an ORF1ab singleplex qPCR assay performed on an exemplary sample containing SARS-CoV-2 nucleic acid, the reagents for the qPCR assay being part of a kit for the detection of SARS-CoV-2 disclosed herein.

[0015] FIG. 2B illustrates the standard curve for the singleplex qPCR assay of FIG. 2A.

[0016] FIG. 2C illustrates the amplification plot of an N protein singleplex qPCR assay performed on an exemplary sample containing SARS-CoV-2 nucleic acid, the reagents for the qPCR assay being part of a kit for the detection of SARS-CoV-2 disclosed herein.

[0017] FIG. 2D illustrates the standard curve for the singleplex qPCR assay of FIG. 2C.

[0018] FIG. 2E illustrates the amplification plot of an S protein singleplex qPCR assay performed on an exemplary sample containing SARS-CoV-2 nucleic acid, the reagents for the qPCR assay being part of a kit for the detection of SARS-CoV-2 disclosed herein.

[0019] FIG. 2F illustrates the standard curve for the singleplex qPCR assay of FIG. 2E.

[0020] FIGs. 3A–3C illustrate comparative amplification plots of ORF1ab (FIG. 3A), N protein (FIG. 3B), and S protein (FIG. 3C) qPCR assays performed on a 7500 Fast Dx instrument (Thermo Fisher Scientific) running the 7500 Standard Protocol or the 7500 Fast Protocol.

[0021] FIGs. 4A–4C illustrate comparative amplification plots of ORF1ab (FIG. 4A), N protein (FIG. 4B), and S protein (FIG. 4C) qPCR assays generated when running identical standard protocols on a 7500 Fast Dx instrument (Thermo Fisher Scientific) or a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific).

[0022] FIGs. 5A–5C illustrate comparative amplification plots of ORF1ab (FIG. 5A), N protein (FIG. 5B), and S protein (FIG. 5C) qPCR assays using the TaqPath 1-Step RT-qPCR Master Mix (Thermo Fisher Scientific) or the TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific) on the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific).

DETAILED DESCRIPTION

[0023] Before describing various embodiments of the present disclosure in detail, it is to be understood that this disclosure is not limited to the parameters of the particularly exemplified systems, methods, apparatus, products, processes, and/or kits, which may, of course, vary. Thus, while certain embodiments of the present disclosure will be described in detail, with reference to specific configurations, parameters, components, elements, etc., the descriptions are illustrative and are not to be construed as limiting the scope of the claimed invention. In addition, the terminology used herein is for the purpose of describing the embodiments and is not necessarily intended to limit the scope of the claimed invention.

[0024] Furthermore, it is understood that for any given component or embodiment described herein, any of the possible candidates or alternatives listed for that component may generally be used individually or in combination with one another, unless implicitly or explicitly understood or stated otherwise. Additionally, it will be understood that any list of such candidates or alternatives is merely illustrative, not limiting, unless implicitly or explicitly understood or stated otherwise.

[0025] In addition, unless otherwise indicated, numbers expressing quantities, constituents, distances, or other measurements used in the specification and claims are to be understood as being modified by the term "about," as that term is defined herein. Accordingly, unless indicated to the contrary, the numerical parameters set forth in the specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained by the subject matter presented herein. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the subject matter presented herein are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical values, however, inherently contain certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

[0026] All publications and patent applications cited herein, as well as the Appendices attached hereto, are incorporated by reference in their entirety for all purposes to the same extent as if each were specifically and individually indicated to be so incorporated by reference. Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the spirit and substance of this disclosure and of the appended claims.

[0027] Any headings and subheadings used herein are for organizational purposes only and are not meant to be used to limit the scope of the description or the claims.

Overview of Compositions, Systems, and Kits for Detection of Target Sequences

[0028] As discussed above, a new variant beta coronavirus, SARS-CoV-2 (also known as 2019-nCoV), has recently emerged as the newest pandemic virus. Current epidemiological data is potentially disadvantaged in view of existing non-specific detection assays used to identify SARS-CoV-2 infections. The lack of a reliable assay for accurately and specifically identifying SARS-CoV-2 from a sample (*e.g.,* a clinical sample obtained from nasopharyngeal swab, nasopharyngeal aspirate, bronchoalveolar lavage, buccal swab, saliva, or urine) and/or differentiating this virus from other common respiratory pathogens may prevent healthcare professionals from properly treating and advising patients. Further, by not being able to accurately and quickly identify individuals infected with SARS-CoV-2, it can be quite difficult to establish a systematic treatment campaign or implement successful preventative measures.

[0029] Given the present and continuing emergence of new coronavirus strains, there is an urgent need to develop methods for the rapid detection and characterization of existing and novel coronavirus strains so that appropriate treatment and infection control measures can be properly instituted in a timely manner. Problematically, many of the available SARS-CoV-2 detection assays are non-specific with respect to detecting and differentiating SARS-CoV-2 from other respiratory pathogens, particularly other coronaviruses, which has potential to lead to a lack of patient confidence in the diagnostic potential of current SARS-CoV-2 detection assays. Further, because individuals infected with SARS-CoV-2 often experience symptoms similar to those infected with Influenza Types A or B and/or Respiratory Syncytial Virus (RSV), and/or other respiratory microbes, there is an additional need to be able to simultaneously test for each of these respiratory infectious agents in order to provide an accurate diagnosis before seeking/providing treatment and/or confining the individual to a quarantined area under the potentially mistaken belief that they are infected with SARS-CoV-2. Each misidentified or misdiagnosed instance of SARS-CoV-2 infection can further convolute the epidemiological data and prevent the implementation of appropriate, informed solutions that may help reign in the pandemic.

[0030] A number of assays are available for allegedly detecting the presence of SARS-CoV-2, such as those provided by the United States Centers for Disease Control and Prevention (US-CDC) that contain probes targeting the N protein, the assay developed by the Chinese CDC targeting the coding regions of the N protein and the ORF1ab protein, and the WHO kit targeting the coding regions of the N protein, the E protein, and the closely related RdRp SARS/Wuhan coronavirus.

While each of the foregoing assays have 100% coverage of all published SARS-CoV-2 genomes to date—meaning these assays are each theoretically capable of identifying the presence of SARS-CoV-2 from a nucleic acid sample—the design of these assays is such that the detection is non-specific, which can perpetuate the issues discussed above instead of alleviating them.

[0031] For example, the probes used in the WHO kit for the E and N proteins map perfectly to hundreds of non-SARS-CoV-2 coronavirus strains. Furthermore, the confirmatory probe identifying RdRp-SARS/Wuhan was designed to detect both SARS and SARS-CoV-2, making it non-specific by design. These assays lack endogenous controls as well. In total, this assay is non-specific for SARS-CoV-2 and is prone to providing false positive results. Similarly, the US-CDC kit for detecting SARS-CoV-2 has also exhibited some non-specificity. It relies on three separate probes to the coding region of the N protein, and two of these probes can generate a false positive signal in the presence of non-SARS-CoV-2 coronaviruses, such as many SARS strains and even the bat-SARS-CoV strain, particularly when present in higher concentrations. Accordingly, even this kit fails to provide an assay having the desired SARS-CoV-2 specificity, and there remains an unmet need in the market for a SARS-CoV-2 detection assay that is accurate and specific and that, preferably, can be implemented quickly with a short turnaround time between obtaining the sample and receiving the results.

[0032] Disclosed herein are compositions, kits, and methods for specifically detecting viral sequences, in particular SARS-CoV-2. Additional compositions, kits, and methods are disclosed that enable the detection and differentiation of SARS-CoV-2 from other related coronaviruses, from respiratory tract microbiota, and from common respiratory pathogens that produce similar symptomatic infections in humans, including Influenza Type A (Flu A), Influenza Type B (Flu B), and Respiratory Syncytial Virus (*e.g.,* RSV A and RSV B). As demonstrated throughout the present description, many of the probes disclosed herein include nucleic acid binding portions that exhibit 100% identity (*i.e.,* no mismatches) to all 52 genomic sequences of SARS-CoV-2 reported in the literature. Further, the kits and methods provided herein specifically target all 71 complete genomes currently available at GISAID, and do not target any of the 2,116 complete genomes of other coronaviruses currently available at NCBI, underscoring the beneficial specificity of the disclosed methods and kits for detecting SARS-CoV-2. Indeed, the disclosed embodiments solve at least some of the unmet needs in the field of viral identification and provide meaningful improvements over prior viral detection compositions, kits, and methods.

[0033] In some embodiments, the strain coverage for the compositions, kits, and methods described herein for detecting SARS-CoV-2, including those with primers and probes selected from SEQ ID NO:4 – SEQ ID NO:2533, is 99.9% based on an *in silico* analysis of 35,833 high

quality complete sequences available from GISAID as of July 6, 2020. Additionally, the compositions, kits, and methods disclosed herein for the detection of viral sequences, particularly those multiplex assays for identifying the specific presence of SARS-CoV-2, Flu A, Flu B, RSV A, and/or RSV B using primers and probes selected from SEQ ID NO:4 – SEQ ID NO:2533, retain the 99.9% specificity and precision for identifying SARS-CoV-2 strains, and the strain coverage is 98.2% (6730/6854) for Flu A and 99.3% (3105/3127) for Flu B, based on data available from NCBI as of April 13, 2020.

[0034] SEQ ID NO:4 – SEQ ID NO:257 includes a list of sequences which are amenable for use as forward primers targeting the ORF1ab, S protein, or N protein coding regions of the SARS-CoV-2 genome, regions of the human influenza (Flu) type A or type B viral genome, regions of the Respiratory Syncytial Virus (RSV) type A or type B viral genome, or control sequences, such as MS2 Phage and RNase P.

[0035] SEQ ID NO:267 – SEQ ID NO:510 includes a list of sequences which are amenable for use as reverse primers targeting the ORF1ab, S protein, or N protein coding regions of the SARS-CoV-2 genome, regions of the human influenza (Flu) type A or type B viral genome, regions of the Respiratory Syncytial Virus (RSV) type A or type B viral genome, or control sequences, such as MS2 Phage and RNase P.

[0036] SEQ ID NO:520 – SEQ ID NO:2533 includes a list of sequences which are nucleic acid portions of probes targeting the ORF1ab, S protein, or N protein coding regions of the SARS-CoV-2 genome, regions of the human influenza (Flu) type A or type B viral genome, regions of the Respiratory Syncytial Virus (RSV) type A or type B viral genome, or control sequences, such as MS2 Phage and RNase P.

[0037] Further, because SARS-CoV-2 is an RNA virus, it can mutate with relatively high frequency, making it difficult to consistently detect over time. Specific detection can be ensured even in the case of future variants by using multiple assays targeting different regions of the same target genes to ensure redundancy and specificity. Unlike other published assay designs, which require multiple assay designs and separate reactions for each loci to enhance specificity, the disclosed primers and probes can differentiate SARS-CoV-2 strains using a single and specific assay conducted in a single, or at the most two, reaction volumes.

[0038] The embodiments of the present disclosure beneficially provide improved compositions, kits, and methods for detecting and differentiating viral respiratory tract pathogens that share similar symptomatic presentations in humans. As such, these disclosed embodiments advantageously improve the efficiency and accuracy of evaluating respiratory samples (*e.g.*, in a

laboratory setting, at point of sale location, and/or at point of care location) for the presence of viral sequences and can improve the diagnosis and treatment of affected individuals.

[0039] The disclosed compositions, kits, and methods for the detection of viral sequences can also improve the accuracy of epidemiological studies related to SARS-CoV-2, Flu A, Flu B, and/or RSV infections. Additional embodiments disclosed herein include assay panels (e.g. in the format of an array card) for determining the presence of viral, bacterial, and fungal nucleic acid sequences that can, among other things, improve syndromic evaluations and epidemiological studies by being able to detect and differentiate SARS-CoV-2 from related coronaviruses, influenza viruses, rhinoviruses, adenoviruses, and other viral, bacterial, and fungal microbes.

Sample Collection

[0040] The disclosed compositions, kits, and methods are configured to detect viral nucleic acid from a sample, preferably a specific and differential detection of SARS-CoV-2 from a sample. The sample may be a veterinary sample (*e.g.,* from non-human animals like mink), a clinical sample (*e.g.,* from a symptomatic or asymptomatic human), a food sample, a forensic sample, an environmental sample (e.g., soil, dirt, garbage, sewage, air, or water), including food processing and manufacturing surfaces, or any other biological sample. In most instances, SARS-CoV-2 or other coronaviruses and respiratory tract pathogens are detected by analysis of swabs or fluid obtained from swabs, such as throat swabs, nasal swabs, nasopharyngeal swabs, nasal mid-turbinate swabs, oropharyngeal swabs, cheek swabs, saliva swabs, or other swabs, though it should be appreciated that SARS-CoV-2 or other coronaviruses and/or respiratory tract pathogens may also be detected by analysis of urine samples, saliva samples, or other clinical samples.

[0041] The sample can be collected by a healthcare professional in a healthcare setting, but in some instances, the sample may also be collected by the subject themselves or by an individual assisting the subject in self-collection. For example, a nasopharyngeal swab has heretofore served as the gold standard for obtaining a sample to be used in clinical diagnostics or screening. Such swabs are often used by a healthcare professional in a healthcare setting. Other samples, such as a saliva sample, can similarly be obtained in a healthcare setting with the assistance or oversight of a healthcare professional. However, in some instances, self-collection of a sample can be more efficient and can be done outside of a healthcare setting.

[0042] In some embodiments, the sample is a raw saliva sample collected—whether by self-collection or assisted/supervised collection—in a sterile tube or specifically-designed saliva collection device. The saliva collection tube/device may be a component of a self-collection kit having instructions for use, such as sample collection instructions, sample preparation or storage

instructions, and/or shipping instructions. In some embodiments, the raw saliva sample can be collected directly into a sealable container without any preservation solution or other fluid or substance in the container prior to receipt of the saliva sample within the container or as a result of closing/sealing the container. In some other embodiments, the raw saliva sample is collected into a container which already contains some amount of a preservation or treatment solution or other fluid or substance.

[0043] Traditionally, a nucleic acid fraction of the sample is extracted or purified from the sample—whether obtained via swab, from raw saliva, or other bodily fluid—prior to any detection of viral nucleic acids therein. Surprisingly, the disclosed embodiments for detecting viral nucleic acid from a sample can be adapted to detect viral nucleic acid directly from a raw saliva sample without a specific nucleic acid purification and/or extraction step prior to its use in downstream detection assays (e.g., RT-qPCR). In some embodiments, the saliva sample is pre-treated prior to use (see, for example, Example 8 herein). This can include, for example, heating the saliva sample, such as by placing the raw saliva sample on a heat block/water bath set to a temperature of 95°C for 30 minutes, followed by combining the heat-treated saliva with a buffer or lysis solution. The buffer or lysis solution can include, for example, any nucleic-acid-amenable buffer such as TBE and may further include a detergent and/or emulsifier such as Triton-X-100, NP-40, or the polysorbate-type nonionic surfactant, Tween-20.

[0044] It should be appreciated that in some embodiments, the disclosed compositions can include the sample mixed with a buffer and detergent/emulsifier. The sample can be added to a buffer/detergent mixture or vice versa. As a non-limiting example, a set of subject samples can be prepared as compositions for downstream analysis and detection of viral sequence by adding a volume of heat-treated sample for each subject into one (or a plurality) of wells in a multi-well plate. A volume of a buffer/detergent mixture (e.g., TBE + Tween-20) can then be added to each well containing a subject sample. Alternatively, a multi-well plate can be loaded with a volume of a buffer/detergent mixture into which a volume of heat-treated saliva is added. Once combined, this probative template solution can be used immediately or stored for later analysis. Such probative template solutions can also be combined with PCR reagents (e.g., buffers, dNTPs, master mixes, etc.) prior to or after storage.

Compositions, Kits, and Methods for Detection of SARS-CoV-2 Viral Sequences

[0045] The primers and probes disclosed herein are useful for the detection of SARS-CoV-2 from a sample, such as a biological sample obtained from a human or non-human (*e.g.,* mink) subject. Such primers and/or probes can be used within a kit for performing a nucleic-acid-based

assay for the detection and identification of one or more target nucleic acids in the sample, which may be single stranded or double stranded of any size. For example, the primers and probes provided in SEQ ID NO:4 – SEQ ID NO:2533 can be used to amplify and/or analyze one or more specific target sequences present in the SARS-CoV-2 viral genome or within one or more of the Flu A, Flu B, RSV A, RSV B, other target respiratory microbes and/or controls (see, e.g., Tables 3A and 3B), as described herein. The amplified products ("amplicons") can be detected and/or analyzed using any suitable method and on any suitable platform.

[0046] Polymerase chain reaction (PCR) and related methods are common methods of nucleic acid amplification. PCR is one, but not the only, example of a nucleic acid polymerase reaction method for amplifying a nucleic acid test sample comprising the use of a known nucleic acid as a primer and a nucleic acid polymerase to amplify or generate a specific target nucleic acid. In general, PCR utilizes a primer pair that consists of a forward primer and a reverse primer configured to amplify a target segment of a nucleic acid template. Typically, but not always, the forward primer hybridizes to the 5' end of the target sequence and the reverse primer will be identical to a sequence present at the 3' end of the target sequence. The reverse primer will typically hybridize to a complement of the target sequence, for example an extension product of the forward primer and/or vice versa. PCR methods are typically performed at multiple different temperatures, causing repeated temperature changes during the PCR reaction ("thermal cycling"). Other amplification methods, such as, e.g., loop-mediated isothermal amplification ("LAMP"), and other isothermal methods, such as those listed in Table 1, may require less or less extensive thermal cycling than does PCR, or require no thermal cycling. Such isothermal amplification methods are also contemplated for use with the assay compositions, reaction mixtures, kits described herein.

Table 1. Summary of optional isothermal amplification methods.

NASBA	Nucleic acid sequence-based amplification (NASBA) is a method used to amplify RNA.
LAMP	Loop-mediated isothermal amplification (LAMP) is a single tube technique for the amplification of DNA. It typically uses 4-6 primers, which form loop structures to facilitate subsequent rounds of amplification.
HDA	Helicase-dependent amplification (HDA) uses the double-stranded DNA unwinding activity of a helicase to separate strands for in vitro DNA amplification at constant temperature.
RCA	Rolling circle amplification (RCA) starts from a circular DNA template and a short DNA or RNA primer to form a long single stranded molecule.

MDA	Multiple displacement amplification (MDA) is a technique that initiates when multiple random primers anneal to the DNA template and the polymerase amplifies DNA at constant temperature.
WGA	When MDA is used to amplify DNA from a whole genome of a cell it is called whole genome amplification (WGA). (Other methods of WGA include MALBAC, LIANTI, DOP-PCR.)
RPA	Recombinase polymerase amplification (RPA) is a low temperature DNA and RNA amplification technique.

[0047] Methods of performing PCR, including those in Table 1, are well known in the art; nevertheless, further discussion of PCR and other methods may be found, for example, in Molecular Cloning: A Laboratory Manual by Green and Sambrook, Cold Spring Harbor Laboratory Press, 4th Edition 2012, which is incorporated by reference herein in its entirety.

[0048] SARS-CoV-2 has a single-stranded positive-sense RNA genome. Other viruses, such as Flu A, Flu B, RSV A, and RSV B also have RNA-based genomes. In some embodiments, therefore, the amplification reaction (e.g., LAMP or PCR) can be combined with a reverse transcription (RT) reaction, such as in RT-LAMP or RT-PCR to convert the RNA genome to a cDNA template. The cDNA template is then used to create amplicons of the target sequences in the subsequent amplification reactions.

[0049] In some embodiments, the amplifying step can include performing qPCR, as that term is defined herein. qPCR is a sensitive and specific method for detecting and optionally quantifying amounts of starting nucleic acid template (e.g., coronaviral nucleic acid) in a sample. Methods of qPCR are well known in the art; one leading method involves the use of a specific hydrolysis probe in conjunction with a primer pair. The hydrolysis probe can include a detectable label (e.g., fluorophore) at one end and a quencher that quenches the detectable label at the other end. In some embodiments, the label is at the 5' end of the probe and cleavage of the 5' label occurs via 5' hydrolysis of the probe by the nucleic acid polymerase as it extends the forward primer towards the probe binding site within the target sequence. The separation of the probe label from the probe quencher via cleavage (or unfolding) of the probe results in an increase in signal which can be detected and optionally quantified. The detectable signal can be monitored over time and analyzed to determine the relative or absolute amount of starting nucleic acid template present in the sample. Suitable labels are described herein. In some embodiments, the dye-quencher combinations are used, such as those described in the Examples. It should be appreciated that qPCR and RT-qPCR methods are known to those having skill in the art. Nevertheless, particular embodiments are

provided in the Examples and provide further details regarding qPCR as well as related compositions and methods of use thereof.

[0050] The reaction vessel or volume can optionally include a tube, channel, well, cavity, site or feature on a surface, or alternatively a droplet (e.g., a microdroplet or nanodroplet) that may be deposited onto a surface or into a surface well or cavity or suspended within (or partially bounded by) a fluid stream. In some embodiments, the reaction volume includes one or more droplets arrayed on a surface or present in an emulsion. The reaction volumes can optionally be formed by fusion of multiple pre-reaction volumes containing different components of an amplification reaction. For example, pre-reaction volumes containing one or more primers can be fused with pre-reaction volumes containing human nucleic acid samples and/or polymerase enzymes, nucleotides, and buffer. In some embodiments involving performing qPCR reactions in array format, a surface contains multiple grooves, channels, wells, cavities, sites, or features defining a reaction volume containing one or more amplification reagents (e.g., primers, probes, buffer, polymerase, nucleotides, and the like). In some array-formatted singleplex embodiments, the reaction volume within the selected tubes, grooves, channels, wells, cavities, sites, or features contains only a single forward primer sequence and a single reverse primer sequence. Optionally, a probe sequence is also included in the singleplex reaction volume.

[0051] In some array-formatted multiplex embodiments, the reaction volume within the selected tubes, grooves, channels, wells, cavities, sites, or features contains multiple (e.g., 2, 3, 4, 5, 6, etc.) forward primer sequences and multiple reverse primer sequences. Optionally, one or more probe sequences is also included in the multiplex reaction volume.

[0052] For instance, exemplary methods for polymerizing and/or amplifying and detecting nucleic acids suitable for use as described herein are commercially available as TaqMan assays (see, e.g., U.S. Patent Nos. 4,889,818; 5,079,352; 5,210,015; 5,436,134; 5,487,972; 5,658,751; 5,210,015; 5,487,972; 5,538,848; 5,618,711; 5,677,152; 5,723,591; 5,773,258; 5,789,224; 5,801,155; 5,804,375; 5,876,930; 5,994,056; 6,030,787; 6,084,102; 6,127,155; 6,171,785; 6,214,979; 6,258,569; 6,814,934; 6,821,727; 7,141,377; and/or 7,445,900, all of which are hereby incorporated herein by reference in their entirety). TaqMan assays are typically carried out by performing nucleic acid amplification on a target polynucleotide using a nucleic acid polymerase having 5'-to-3' nuclease activity, a primer capable of hybridizing to the target polynucleotide, and an oligonucleotide probe capable of hybridizing to said target polynucleotide 3' relative to the primer. The oligonucleotide probe typically includes a detectable label (e.g., a fluorescent reporter molecule) and a quencher molecule capable of quenching the fluorescence of the reporter molecule. Typically, the detectable label and quencher molecule are part of a single probe. As

amplification proceeds, the polymerase digests the probe to separate the detectable label from the quencher molecule. The detectable label is monitored during the reaction, where detection of the label corresponds to the occurrence of nucleic acid amplification (e.g., the higher the signal the greater the amount of amplification). Variations of TaqMan assays are known in the art and would be suitable for use in the methods described herein.

[0053] For example, a singleplex or multiplex qPCR can include a single TaqMan dye associated with a locus-specific primer or multiple TaqMan dyes respectively associated with a plurality of loci in a multiplex format. As a non-limiting example, a 4-plex reaction can include FAM (emission peak ~517 nm), VIC (emission peak ~551 nm), ABY (emission peak ~580 nm), and JUN (emission peak ~617 nm) dyes, each dye being associated with a different target sequence and each dye being quenched by QSY, can allow up to 4 targets to be amplified and tracked real-time within a single reaction vessel. These aforementioned reporter dyes are optimized to work together with minimal spectral overlap for improved performance. These dyes can additionally be combined with Mustang Purple (emission peak ~654 nm) for use monitoring fluorescence of a control or for use in a non-emission-spectrum-overlapping 5-plex assay. In addition, the QSY quencher is fully compatible with probes that have minor-groove binder quenchers.

[0054] Detector probes may be associated with alternative quenchers, including without limitation, dark fluorescent quencher (DFQ), black hole quenchers (BHQ), Iowa Black, QSY quencher, and Dabsyl and Dabcel sulfonate/carboxylate Quenchers. Detector probes may also include two probes, wherein, for example, a fluorophore is associated with one probe and a quencher is associated with a complementary probe such that hybridization of the two probes on a target quenches the fluorescent signal or hybridization on the target alters the signal signature via a change in fluorescence. Detector probes may also include sulfonate derivatives of fluorescein dyes with SO_3 instead of the carboxylate group, phosphoramidite forms of fluorescein, phosphoramidite forms of Cy5.

[0055] It should be appreciated that when using more than one detectable label, particularly in a multiplex format, each detectable label should differ in its spectral properties from the other detectable labels used therewith such that the labels may be distinguished from each other, or such that together the detectable labels emit a signal that is not emitted by either detectable label alone. Exemplary detectable labels include, for instance, a fluorescent dye or fluorophore (e.g., a chemical group that can be excited by light to emit fluorescence or phosphorescence), "acceptor dyes" capable of quenching a fluorescent signal from a fluorescent donor dye, and the like, as described above. Suitable detectable labels may include, for example, fluoresceins (e.g., 5-carboxy-2,7-dichlorofluorescein; 5-Carboxyfluorescein (5-FAM); 5-Hydroxy Tryptamine (5-HAT); 6-JOE; 6-

carboxyfluorescein (6-FAM); Mustang Purple, VIC, ABY, JUN; FITC; 6-carboxy-4',5'-dichloro-2',7'-dimethoxy¬fluorescein (JOE)); 6-carboxy-1,4-dichloro-2',7'-dichloro¬fluorescein (TET); 6-carboxy-1,4-dichloro-2',4',5',7'-tetra-chlorofluorescein (HEX); Alexa Fluor fluorophores (e.g., 350, 405, 430, 488, 500, 514, 532, 546, 555, 568, 594, 610, 633, 635, 647, 660, 680, 700, 750); BODIPY fluorophores (e.g., 492/515, 493/503, 500/510, 505/515, 530/550, 542/563, 558/568, 564/570, 576/589, 581/591, 630/650-X, 650/665-X, 665/676, FL, FL ATP, FI-Ceramide, R6G SE, TMR, TMR-X conjugate, TMR-X, SE, TR, TR ATP, TR-X SE), Cascade Blue, Cascade Yellow; Cy™ dyes (e.g., 3, 3.18, 3.5, 5, 5.18, 5.5, 7), cyan GFP, cyclic AMP Fluorosensor (FiCRhR), fluorescent proteins (e.g., green fluorescent protein (e.g., GFP. EGFP), blue fluorescent protein (e.g., BFP, EBFP, EBFP2, Azurite, mKalama1), cyan fluorescent protein (e.g., ECFP, Cerulean, CyPet), yellow fluorescent protein (e.g., YFP, Citrine, Venus, YPet), FRET donor/acceptor pairs (e.g., fluorescein/fluorescein, fluorescein/tetramethylrhodamine, IAEDANS/fluorescein, EDANS/dabcyl, BODIPY FL/BODIPY FL, Fluorescein/QSY7 and QSY9), LysoTracker and LysoSensor (e.g., LysoTracker Blue DND-22, LysoTracker Blue-White DPX, LysoTracker Yellow HCK-123, LysoTracker Green DND-26, LysoTracker Red DND-99, LysoSensor Blue DND-167, LysoSensor Green DND-189, LysoSensor Green DND-153, LysoSensor Yellow/Blue DND-160, LysoSensor Yellow/Blue 10,000 MW dextran), Oregon Green (e.g., 488, 488-X, 500, 514); rhodamines (e.g., 110, 123, B, B 200, BB, BG, B extra, 5-carboxytetramethylrhodamine (5-TAMRA), 5 GLD, 6-Carboxyrhodamine 6G, Lissamine, Lissamine Rhodamine B, Phallicidine, Phalloidine, Red, Rhod-2, ROX (6-carboxy-X-rhodamine), 5-ROX (carboxy-X-rhodamine), Sulphorhodamine B can C, Sulphorhodamine G Extra, TAMRA (6-carboxytetramethyl¬rhodamine), Tetramethylrhodamine (TRITC), WT), Texas Red, Texas Red-X, among others as would be known to those of skill in the art.

[0056] Other detectable labels may also be used. For example, primers can be labeled and used to both generate amplicons and to detect the presence (or concentration) of amplicons generated in the reaction, and such may be used in addition to or as an alternative to labeled probes described herein. As a further example, primers may be labeled and utilized as described in Nazarenko et al. (Nucleic Acids Res. 2002 May 1; 30(9): e37), Hayashi et al. (Nucleic Acids Res. 1989 May 11; 17(9): 3605), and/or Neilan et al. (Nucleic Acids Res. Vol. 25, Issue 14, 1 July 1997, pp. 2938–39). Those of skill in the art will also understand and be capable of utilizing the PCR processes (and associated probe and primer design techniques) described in Zhu et al. (Biotechniques. 2020 Jul: 10.2144/btn-2020-0057).

[0057] In some embodiments, intercalating labels can be used such as ethidium bromide, SYBR Green I, SYBR GreenER, and PicoGreen (Life Technologies Corp., Carlsbad, CA), thereby

allowing visualization in real-time, or end point, of an amplification product in the absence of a detector probe. It should be appreciated, however, that use of intercalating labels may limit multiplexing capabilities, as many intercalating labels are non-specific for a given sequence and merely report the total (or proportional) nucleic acid content within a reaction. In some embodiments, real-time visualization may include both an intercalating detector probe and a sequence-based detector probe. The detector probe can be at least partially quenched when not hybridized to a complementary sequence in the amplification reaction and is at least partially unquenched when hybridized to a complementary sequence in the amplification reaction. In some embodiments, probes may further comprise various modifications such as a minor groove binder to further provide desirable thermodynamic characteristics.

[0058] The genetic sequence of SARS-CoV-2 is available as NCBI accession no. NC_045512.2 and as GenBank accession no. MN908947.3, describing a positive-sense, single-stranded RNA genome of 29,844 base pairs; occasionally, such sequence is referred to herein as the 'normal', 'wild type' or 'reference' sequence for SARS-CoV-2, as opposed to SARS-CoV-2 variant or mutant sequences. Initial genetic characterizations of SARS-CoV-2 identified three coronaviruses having close homology to SARS-CoV-2, namely Bat-SL-CoVZC45, Bat-SL-CoVZXC21, and SARS-CoVGZ02. The sequence identity between these strains is depicted in FIGs. 1A and 1B. In particular, FIG. 1A illustrates the sequence identity between the consensus SARS-CoV-2 sequence as compared to each of Bat-SL-CoVZC45, Bat-SL-CoVZXC21, and SARS-CoVGZ02, across the complete genome as well as across each specific gene region within the coronavirus genome. FIG. 1B illustrates the tabular information of FIG. 1A in graphical form with the x-axis being the base pair position within the viral genome and the y-axis being the percent similarity of each related virus to the corresponding SARS-CoV-2 consensus sequence.

[0059] The analysis illustrated in FIGs. 1A and 1B identified at least three genetic regions with significant variability between SARS-CoV-2 and the other related viruses, specifically within the viral genes encoding the ORF1ab protein (SEQ ID NO:1; base pair 1 corresponds to base pair 1000 of MN908947.3), the S protein (SEQ ID NO:2; base pair 1 corresponds to base pair 21564 of MN908947.3), and the N protein (SEQ ID NO:3; base pair 1 corresponds to base pair 28275 of MN908947.3). The region comprising the coding sequence for the ORF1ab protein is between base pairs 1000–3000 of the SARS-CoV-2 genome; this sequence corresponds to SEQ ID NO:1. The 2,000 base pair region of the SARS-CoV-2 genome that includes the coding sequence for the S protein is between base pairs 21,564–23,564; this sequence corresponds to SEQ ID NO:2. Finally, the 1,283 base pair region of the SARS-CoV-2 genome that includes the coding sequence for the N protein is between base pairs 28,275–29,558; this sequence corresponds to SEQ ID NO:3.

[0060] In some embodiments, detecting amplification of target sequences includes measuring one or more signals emitted by a detectable label attached to, or associated with, one or more primers or probes. Optionally, the one or more signals are measured multiple times as the amplification reaction progresses, in some embodiments at least once per thermal cycle (e.g. during or just after an annealing or extension phase of a thermal cycle), thus allowing for amplification to be detected in 'real time'. In 'multiplex' amplification embodiments, the formation of a plurality of separate and different amplification products can be tracked over time by measuring a signal in one or more detection channels. The signal can be emitted by a detectable label, optionally a fluorescent label, attached to a primer and/or probe that selectively hybridizes to the amplification product. In some embodiments, each channel is calibrated to preferentially or selectively detect a corresponding amplification product and the signal in each channel is used as a measure of concentration of the corresponding amplification product. For example, in some embodiments, an amplification product of the S gene from SARS-CoV-2 is detected in a first detection channel based on a first signal emitted by a first label attached to, or associated with, a first primer and/or first probe that selectively hybridizes to the S gene amplification product, and an amplification product of the N gene is detected in a second detection channel based on a second signal emitted by a second label attached to, or associated with, a second primer and/or second probe that selectively hybridizes to the N gene amplification product. Optionally, in 'triplex' embodiments involving amplification of the Orf1ab gene, the amplification product of the ORF1ab gene is detected in a third channel based on a third signal emitted by a third label attached to a third primer and/or third probe that selectively hybridizes to the ORF1ab amplification product or to the ORF1ab target region. In some embodiments, the amplification product of a control or reference sequence is detected in a fourth channel based on a fourth signal emitted by a fourth label attached to a fourth primer and/or fourth probe that selectively hybridizes to an amplification product of, or to a target sequence within the control or reference sequence.

[0061] In some embodiments (e.g., in the well-known and widely used TaqMan™ line of qPCR assays), detecting an amplification product includes detecting a signal emitted by a fluorescent label attached to the 5' end of a cleavable probe that selectively hybridizes to the amplification product during amplification. The cleavable probe further includes a quencher that quenches the fluorescent label to a 'baseline' fluorescence level. The 5' end of the cleavable probe is cleaved by the polymerase during the extension step, resulting in the separation of the fluorescent label from the quencher and a corresponding increase in fluorescence over baseline. As the PCR reaction progresses, the continuing increase in fluorescence over baseline is measured at each cycle. In some embodiments, an amplification product from the N gene of SARS-CoV-2 is

detected in a first channel based on a first signal emitted by the VIC dye attached to a probe that selectively hybridizes to the corresponding amplification product from the N gene. Optionally, a second amplification product from the S gene of SARS-CoV-2 is detected in a second channel based on a second signal emitted by the ABY dye attached to a probe that selectively hybridizes to the corresponding amplification product from the S gene. Optionally, a third amplification product from the ORF1ab gene of SARS-CoV-2 is detected in a third channel based on a third signal emitted by the FAM dye attached to a probe that selectively hybridizes to the corresponding amplification product from the ORF1ab gene. Optionally, a fourth amplification product from a control or reference sequence is detected in a fourth channel based on a fourth signal emitted by the JUN dye attached to a probe that selectively hybridizes to the corresponding amplification product from the control or reference sequence.

[0062] In some embodiments, a passive reference dye, such as ROX™ is included in the reaction mixture. The metric "Rn" is optionally used to track progress of the amplification reaction and to determine the amount of target sequence originally present in the reaction mixture prior to amplification. Rn can be calculated as the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye present in the reaction mixture; i.e., Rn is the reporter signal normalized to the fluorescence signal of the passive reference dye. In some embodiments, Rn is plotted against PCR cycle number. In some embodiments, ΔRn (calculated as Rn minus the baseline) can be plotted against PCR cycle number. In some embodiments, an amplification plot shows the variation of log (ΔRn) with PCR cycle number. C_t (threshold cycle) is the intersection between an amplification curve and a threshold line. The lower the Ct value for a given amplification product, the earlier the amplification is detectable and the higher the absolute amount, and the relative concentration, of the corresponding target sequence originally present in the reaction mixture. In some embodiments, cutoffs for Ct are used to determine whether a target sequence was originally present or absent in the reaction mixture prior to amplification. For example, in some embodiments a target sequence is determined to be present if the Ct value is less than or equal to 37.

[0063] In some embodiments, emerging variants of SARS-CoV-2 are detectable even if such variants include mutations in one or more of the target regions described above (*i.e.*, ORF1ab protein, S protein, or N protein regions). By looking at multiple target regions within the SARS-CoV-2 genome, accurate detection is achievable even in situations where mutations are significant enough to lead to a negative test result in one (or even two) of the target regions. For example, newly emerging variant B.1.1.7 (often referred to as "the UK variant") has an unusual number of mutations associated with the S protein region. These mutations are substantial enough that some

test components and protocols designed for earlier SARS-CoV-2 variants will show a negative result for the S protein region. However, the built-in redundancy of looking at multiple regions ensures that the overall test is still capable of detecting the SARS-CoV-2 variant B.1.1.7 (based on positive ORF1ab and/or N protein region detection) without significant effects on the overall accuracy of the test. In another example, the 501Y.V2 variant (discovered in South Africa) has not been found to affect detection of the S protein region or any of the other tested regions described herein. Nevertheless, the robustness and redundancy of embodiments that target multiple regions of the SARS-CoV-2 genome limit the risk that these variants, or others that emerge in the future, will significantly impact the overall accuracy of SARS-CoV-2 detection.

[0064] To maximize the specificity of a genetic assay for SARS-CoV-2, primers and probes were designed that targeted the coding regions for ORF1ab, S protein, and N protein. In particular, the viral detection kits, arrays, assays, etc. disclosed herein include primers and/or probes specific for the SARS-CoV-2 genetic sequence encoding the S protein; none of the SARS-CoV-2 viral detection kits currently available target the S gene. Targeting the S gene (in addition to the coding regions associated with the N protein and ORF1ab) provides several advantages in terms of specificity and reliability. For example, at least some of the disclosed assays targeting the coding region for the S protein have been shown to differentiate between SARS-CoV and SARS-CoV-2 at the receptor binding level. Inclusion of these primers and/or probes targeting the S gene sequences offers higher specificity in detection of SARS-CoV-2 strains against other similar coronaviruses, especially in geographical regions where subjects present with co-infections of SARS-CoV and SARS-CoV-2.

[0065] The specificity of the primers and probes provided in SEQ ID NO:4 – SEQ ID NO:2533 was estimated *in silico* using the standard mapping algorithm zpcr3p. These primers and probes were found to exhibit higher specificity for SARS-CoV-2 *in silico* than the primers and probes of other commercially available SARS-CoV-2 qPCR-based assays. Accordingly, the disclosed compositions, kits, and methods for detecting viral sequences include at least one primer and/or probe having a sequence defined by SEQ ID NO:4 – SEQ ID NO:2533, which enables the singleplex and multiplex assays described herein to demonstrate a high level of sensitivity, specificity, and accuracy. In some embodiments, a first forward primer is SEQ ID NO: 160. In some embodiments, a second forward primer is SEQ ID NO: 100. In some embodiments, a third forward primer is SEQ ID NO: 211. In some embodiments, a first reverse primer is SEQ ID NO: 468. In some embodiments, a second reverse primer is SEQ ID NO: 337. In some embodiments, a third reverse primer is SEQ ID NO: 501 and/or 510. In some embodiments, the probes are SEQ ID NOs: 1049, 864 and/or 833. In some embodiments of the disclosed compositions, kits, and

methods, the first forward primer is SEQ ID NO: 160, the second forward primer is SEQ ID NO: 100, the third forward primer is SEQ ID NO: 211, the first reverse primer is SEQ ID NO: 468, the second reverse primer is SEQ ID NO: 337, the third reverse primer is SEQ ID NO: 501 and/or 510, and the complementary probes are SEQ ID NOs 1049, 864 and 833, respectively.

[0066] For example, the sensitivity for the assays described herein can be at least 30 GCE/rxn, 25 GCE/rxn, 20 GCE/rxn, 15 GCE/rxn, or 10 GCE/rxn, including all ranges and numbers in between, for simultaneous detection of multiple targets or pathogens including any of those described herein. In some embodiments, the multiplex assays described herein demonstrate a linear dynamic range (LDR) of detection from 10^7 to 10 GCE/rxn when calculated from a serial dilution. As used herein, "linear dynamic range (LDR)" refers to the range of input template (between the highest and lowest input RNA or DNA) for which acceptable linearity (R2 is $\geq$ 0.980) and efficiency (preferably between 90–110%) are observed.

[0067] In some embodiments, the singleplex assays described herein can demonstrate a sensitivity level down to 1–10 copies/μL input per reaction. For example, the sensitivity for the assays described herein can be as low as 20 copies/μL, 15 copies/μL, 10 copies/μL, 5 copies/μL, 4 copies/μL, 3 copies/μL, 2 copies/μL or 1 copy/μL per reaction, including all numbers and ranges in between. In some embodiments, the singleplex assays described herein can demonstrate an LDR over a range of at least 5 to 6 orders of magnitude with an R2 > 0.99 and a PCR efficiency near 100% using serial dilutions. For example, the singleplex assays described herein can demonstrate an LDR increase of at least 10^3, 10^4, 10^5, or 10^6. In some embodiments, a sample input range from 10^7 down to 10^1 copies, by serial dilution, is linear using the assays described herein.

[0068] In some embodiments, amplification of RNA viral genomes is achieved by performing reverse transcription followed by amplification of at least a portion of the resultant cDNA. Suitable methods are known in the art and include, for example, RT-PCR or RT-LAMP methods where the target sequence (*e.g.,* viral RNA genome) is reverse transcribed to form a first cDNA strand, which is then copied in a template-dependent fashion to form a double stranded DNA sequence. The target sequence is then amplified from this double-stranded cDNA.

[0069] In some embodiments, RT-PCR is performed using samples comprising virus particles or suspected of comprising virus particles, which may be infectious virions, non-infectious or inactivated viral capsids enclosing the viral nucleic acid, or viral genomic RNA obtained from an infected cell. In such embodiments, the compositions, reaction mixtures, and kits disclosed herein can include at least one RNA-dependent DNA polymerase, generally termed a reverse transcriptase (RT), and related components for carrying out reverse transcription. RT-PCR may be performed

using the compositions, reaction mixtures, and kits described herein, when, for example, RNA is the starting material for subsequent analysis.

[0070] In some embodiments, the RT-PCR may be a one-step procedure using one or more primers and one or more probes as described herein. In some embodiments, the RT-PCR may be carried out in a single reaction tube, reaction vessel (e.g., "single-tube" or "1-tube" or "single-vessel" reaction). In some embodiments, the RT-PCR may be carried out in a multi-site reaction vessel, such as a multi-well plate or array. In some embodiments, RT and PCR are performed in the same reaction vessel or reaction site, such as in 1-step or 1-tube RT-qPCR. Suitable exemplary RTs can include, for instance, a Moloney Murine Leukemia Virus (M-MLV) Reverse transcriptase, SuperScript Reverse Transcriptases (Thermo Fisher Scientific), SuperScript IV Reverse Transcriptases (Thermo Fisher Scientific), or Maxima Reverse Transcriptases (Thermo Fisher Scientific), or modified forms of any such RTs.

[0071] In some embodiments, only a single RT-qPCR assay (consisting of a given forward primer and a given reverse primer sequence) is included within a reaction vessel or volume, a reaction mode referred to as "singleplex" herein. Optionally, the singleplex qPCR assay can also include a single probe sequence in addition to the forward primer sequence and the reverse primer sequence. The probe sequence can be a hydrolysis probe sequence. Optionally, the probe includes an MGB (minor groove binding protein), as in TaqMan probes.

[0072] In some embodiments, the disclosed compositions and methods can be used in multiplex format, wherein two or more qPCR assays, each capable of amplifying and detecting a different target sequence, are present in a single reaction volume. In some embodiments, different assays in the same reaction volume will cause a corresponding different amplification product to be generated when the reaction volume is subjected to appropriate amplification conditions and multiple amplicons may be formed in the same reaction volume. The different amplification products can be produced simultaneously when the reaction volume is subjected to amplification conditions; alternatively, different amplification products may be produced serially or consecutively. For example, some assay reaction products may take longer to appear than others due to initial starting concentration of template or may benefit from different reaction conditions for optimal production.

[0073] In some embodiments, different assay products can be independently detected or at least discriminated from each other. For example, different assay products may be distinguished optically (*e.g.,* using optically different labels for each qPCR assay whose emission spectra can be, for example, on the light spectrum, inclusive of infrared, UV, and visible light) or can be discriminated using some other suitable method, including as described in U.S. Patent Publication

No. 2019/0002963, which is incorporated herein by reference in its entirety. In some embodiments, specific combinations of labels are used to differentiate between different pathogens, strains, and/or types of pathogens. For example, different respiratory pathogens or viruses may be differentiated from one another using different labels specific to each pathogen or virus such that the label is detectable only in the presence—and amplification—of the pathogen- or viral-specific nucleic acid sequence.

[0074] In some array-based embodiments, two or more different qPCR assays (each containing a forward primer, a reverse primer and optionally a probe) are present in a single well, cavity, site or feature of the array and products of each assay can be independently detected. For example, different assay products may be discriminated optically (e.g., using different labels present as components of each assay) or using some other suitable method, including as described in U.S. Patent Publication No. 2019/0002963. In some embodiments, at least one primer of each assay contains an optically detectable label that can be discriminated from the optical label of at least one other assay. For the purposes of this disclosure, a PCR assay, which for the sake of clarity is inclusive of any polymerase-driven amplification reaction disclosed herein (*e.g.,* qPCR and RT-qPCR), is considered different from another PCR assay if the respective amplicons differ in nucleic acid sequence by at least one nucleotide.

[0075] In a preferred multiplex format, at least two different assays are combined into a single reaction volume to determine at least the presence of SARS-CoV-2 from a nucleic acid sample obtained or derived from a clinical or laboratory source.

[0076] It should be appreciated that the subject and type of sample may vary. For example, a nasopharyngeal swab has traditionally served as the gold standard in many clinical diagnostic situations, particularly those associated with upper respiratory tract infections. A nucleic acid fraction of the sample obtained by the nasopharyngeal swab can be extracted and used for downstream analysis, such as RT-qPCR. The swab (or other samples disclosed herein) may be obtained or collected from a human subject, but it should be appreciated that the disclosed embodiments can additionally extend to the processing of samples from non-human subjects, such as non-human animals. In some embodiments, the non-human animal subject can be a mink or other domesticated (or non-domesticated) animal, and the disclosed compositions, kits, and methods can be used to detect the presence of SARS-CoV-2 within the non-human animal (*e.g.,* for diagnostic or screening purposes).

[0077] As an additional example, the collected sample may be a raw saliva sample. As provided herein, the raw saliva sample can be self-collected (e.g., within a saliva collection device or sterile tube) or collected from the subject by any other individual in proximity to the subject. In

some embodiments, the raw saliva sample is collected directly into a sealable container without any preservation solution or other fluid or substance in the container prior to receipt of the saliva sample or as a result of closing/sealing the container. The disclosed embodiments for detecting viral nucleic acid from a sample can be adapted to detect viral nucleic acid directly from the saliva sample, or in alternative embodiments, the sample can undergo a specific RNA purification and/or extraction step prior to its use in a detection assay (e.g., RT-qPCR). Thus, it should be appreciated that in some embodiments, a subject sample (e.g., saliva) can directly serve as sample input for subsequent downstream analyses, such as PCR, and this can be accomplished, in some embodiments, with no nucleic acid purification and/or extraction step prior to its use.

[0078] In some embodiments, a method for detecting viral nucleic acid, particularly SARS-CoV-2, directly from a raw saliva sample can include collecting or receiving a saliva sample from a subject and heat treating the sample such as by placing the raw saliva sample on a heat block/water bath set to a temperature of 95°C for 30 minutes. The heating step can provide many benefits, including, for example, denaturing nucleases such as RNase within the saliva that may interfere with accurate assessments of viral presence. Heating the raw saliva sample can also break down the mucus, making the sample more amenable to manipulation with laboratory equipment such as pipettes. The high heat can also cause thermal disruption of any prokaryotic and eukaryotic cells present in the sample and can also disrupt enveloped viruses and/or viral capsids present in the sample and thereby increase accessibility to any viral nucleic acid.

[0079] The method can additionally include mixing the heat-treated sample (e.g., via vortexing the sample for at least 10 seconds) before and/or after equilibrating the heat-treated sample to room temperature. A lysis solution can then be prepared and combined (e.g., in 1:1 proportions) with the heat-treated sample to create a probative template solution for detecting the presence of viral nucleic acid within the sample via nucleic acid amplification reactions (e.g., PCR, RT-PCR, qPCR, RT-qPCR, or the like). The lysis solution can include a nucleic-acid-amenable buffer such as TBE combined with a detergent and/or emulsifier (*e.g.,* Tween-20, Triton-X-100, NP-40, or the like), the polysorbate-type nonionic surfactant. The detergent and/or emulsifier can promote better mixing of the reagents and may also act to increase accessibility to any viral nucleic acid within the sample (e.g., by removing lipid envelopes from virions).

[0080] Once the probative template solution is formed, it can be combined with PCR reagents and subjected to conditions suitable to generate viral-specific (e.g., SARS-CoV-2-specific, Flu A/B-specific, and/or RSV-A/B-specific) amplicons if the viral nucleic acid is present. In some embodiments, the primers can be selected from SEQ ID NO:4 – SEQ ID NO:510 and can be coupled with one or probes generated from sequences disclosed in SEQ ID NO:520 – SEQ ID

NO:2533 to specifically identify the amplified coding regions associated with the SARS-CoV-2 N protein and S protein and/or ORF1ab region in addition to any other selected viral sequence to be identified. In some embodiments, the primers and/or probes can be included within a kit, which may additionally include an internal control primer and probe set for identifying a positive control coding sequence (*e.g.,* endogenously present human RNase P RPP30 sequence). The foregoing and similar methods are beneficially compatible with some currently available assays/kits, such as the TaqCheck SARS-CoV-2 Fast PCR Assay, TaqCheck SARS-CoV-2 Control, and TaqCheck SARS-CoV-2 Control Dilution Buffer, and may additionally be compatible with preexisting thermal cycling master mixes, such as TaqPath 1-Step RT-qPCR Master Mix, CG.

[0081] Notably, in some embodiments, the nucleic acid amplification protocol can be configured for rapid processing (e.g., in less than about 45 minutes) and high throughput, allowing for a minimally invasive method to quickly screen large numbers of individuals in a scalable way. This can be particularly useful to perform asymptomatic testing (e.g., high frequency/widespread testing at schools, workplaces, conventions, sporting events, large social gatherings, etc.) or for epidemiological purposes. The disclosed embodiments can also beneficially provide a lower cost sample collection system and method that enables self-collection (reducing health care professional (HCP) staffing needs) using a low-cost collection device. This eliminates the requirements for swabs, buffers, virus transmission media (or other specialized transport medium), and the like. The disclosed embodiments also allow for a reduction in Personal Protective Equipment (PPE) requirements and costs. Because the reagents and methods are streamlined (e.g., no precursor nucleic acid purification and/or extraction step), there is a reduced use of nucleic acid preparation plastics which brings a coincident reduction in reagent costs and inventory costs. There is also a beneficial reduced dependence on supply-constrained items, and the compatibility of these methods and kit components with existing equipment improves the flexibility and simplicity of their implementation to the masses. Overall, such embodiments allow for a less expensive assay that can be accomplished more quickly from sample collection through result generation.

[0082] The disclosed methods for the direct detection of viral nucleic acids, particularly SARS-CoV-2, from a raw saliva sample are beneficially robust. For example, the probative template obtained from the raw saliva sample can be used and/or is compatible with many varied PCR reagents and/or commercially available kits. That is, in some embodiments, a saliva sample that is received directly from the subject (whether through self-collection or assisted collection), heat treated, and combined with a buffer/detergent mixture (*e.g.,* a TBS/Tween-20 solution) for use as the sample input for existing PCR/LAMP kits and is otherwise compatible with many commercially available PCR/LAMP buffers, polymerases, and other PCR/LAMP reagents. Indeed,

in some embodiments, no additional steps or modifications to the existing PCR/LAMP protocols are needed when using the heat-treated saliva sample in TBS/Tween-20 (or other buffer/detergent mixture) as the nucleic acid source for amplification. This allows for rapid adoption and implementation while maintaining the desired (and often requisite) specificity and reliability of a diagnostic test. In some embodiments, the disclosed methods for the direct detection of SARS-CoV-2 from a raw saliva sample has a sensitivity of <10,000 GCE/mL. In some embodiments, the methods and kits have a sensitivity of <5,000 GCE/mL, <2,500 GCE/mL, <1,000 GCE/mL, or any value or range therebetween. For example,

[0083] An exemplary method for detecting viral nucleic acid, particularly SARS-CoV-2, directly from a raw saliva sample is provided in the Examples section below.

[0084] As provided herein, it should be appreciated that the nucleic acid sample can obtained from other body tissues or sources, including but not limited to bodily fluids other than saliva (e.g., blood, urine, sputum, and the like). In some embodiments, SARS-CoV-2 or other viruses are detected by analysis of swabs, or fluid obtained from swabs, such as throat swabs, nasal swabs, nasopharyngeal swabs, cheek swabs, saliva swabs, or other swabs. In some embodiments, the nucleic acid sample includes a total nucleic acid content isolated from a subject via nasopharyngeal swab, nasopharyngeal aspirate, and/or bronchoalveolar lavage. However, it should be appreciated that SARS-CoV-2 or other coronaviruses and/or other viruses may also be detected by analysis of urine samples, saliva samples, or other suitable clinical samples.

[0085] In some embodiments, particularly where the sample is not used directly to identify the presence of viral sequences, an initial step for detecting viral sequences can include subjecting each sample to a nucleic acid purification assay. The purified or otherwise extracted nucleic acid fraction of the subject sample is then added to the downstream detection assays. The total nucleic acid content can be isolated by any means known in the art, including, for example, using a MagMAX Viral/Pathogen Ultra Nucleic Acid Isolation Kit (sold by Thermo Fisher Scientific under Cat. No. A42356). In short, the MagMAX kit provides nucleic-acid-binding beads and other reagents for binding, washing, and eluting nucleic acid from a clinical/laboratory sample. These eluted nucleic acids preferably include the total nucleic acid content purified from the sample.

[0086] In some embodiments, the total nucleic acid content includes any genomic DNA and transcribed RNA derived from subject cells captured within the sample in addition to any genomic/transcribed nucleic acids derived from the subject's eukaryotic and/or prokaryotic microbiota captured during the sampling process. Any single-stranded (plus or minus) RNA, double-stranded RNA, cDNA derivatives thereof, single-stranded (plus or minus) DNA, or double-stranded DNA derived from viruses captured in the sample are also, preferably, included within

the total nucleic acid content eluted from the clinical/laboratory sample. This eluted total nucleic acid content can then be used as the source of template nucleic acid within the disclosed RT-qPCR assays.

[0087] The compositions, reaction mixtures, and kits may also comprise any other components necessary for carrying out such RT-qPCR reactions, such as may be found in SuperScript IV VILO Master Mix (Thermo Fisher Scientific), TaqPath 1-Step RT-qPCR Master Mix (Thermo Fisher Scientific), TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific) or any other suitable RT-PCR master mixes which may be commercially available. RT-PCR protocols are known within the art. Notwithstanding this, an exemplary RT-PCR protocol for use in the multiplex viral detection protocols described herein is set forth in the Examples.

[0088] In some embodiments, the reverse transcription and/or nucleic acid amplification assays as described herein are performed using a real-time quantitative PCR (qPCR) instrument, including for example a QuantStudio Real-Time PCR system, such as the QuantStudio 5 RealTime PCR System (QS5) and QuantStudio 12K Flex System (QS12K), or a 7500 Real-Time PCR system, such as the 7500 Fast Dx system, from Thermo Fisher Scientific.

[0089] The disclosed kits for detecting viral sequences contain, in some embodiments, one or more of the forward primers, reverse primers, and/or probes for detecting target nucleic acid sequences in the SARS-CoV-2 genome, such as disclosed in SEQ ID NO:4 – SEQ ID NO:251; SEQ ID NO:267 – SEQ ID NO:504 and SEQ ID NO:510; and SEQ ID NO:520 – SEQ ID NO:1295 and SEQ ID NO:1466 – SEQ ID NO:2359, respectively. In some embodiments, the primers described herein are included in the kits, array cards, and similar at a concentration from about 100 nM to 1 mM (e.g. 300nM, 400nM, 500 nM, etc.), including all concentration amounts and ranges in between. In some embodiments, the probes described herein are used in a nucleic acid assay at a concentration from about 50 nM to 500 nM (e.g., 75 nM, 125 nM, 250 nM, etc.), including all concentration amounts and ranges in between.

[0090] The disclosed kits, arrays, etc. disclosed herein can include reagents for performing a nucleic acid amplification method, as discussed herein, disclosed herein typically include at least one primer pair (e.g., a forward primer selected from SEQ ID NO:4 – SEQ ID NO:251 and a reverse primer selected from SEQ ID NO:267 – SEQ ID NO:504 and SEQ ID NO:510) directed to a SARS-CoV-2 target nucleic acid sequence, a nucleic acid polymerase (*e.g.,* temperature-tolerant DNA-dependent DNA polymerases such as Taq or RNA-dependent DNA polymerases, also known as reverse transcriptase), and a pool of deoxynucleotide triphosphates (dNTPs) for synthesizing cDNA templates and/or amplicons. Each of the foregoing can be included individually within the disclosed kits, as needed, in concentrations suitable for the various thermal

cycling reactions in which they are used, as known in the art. In some embodiments, the kits can additionally include buffers and/or salts at an appropriate concentration for the generation of a reaction mixture that, for example, increases levels of polymerase activity in the reaction mixture, as known in the art or otherwise disclosed herein.

[0091] In some embodiments, optimal amplification and detectability for viral genomes is achieved by the disclosed kits using a master mix included therein and which can be added to the reaction volume prior to amplification. A master mix can include, for example, one or more of a nucleic acid polymerase, dNTPs, buffers, and salts—all of which are included in the master mix at the appropriate concentration such that when the desired volume of sample is added (with or without balancing volumes of PCR-grade water). In some embodiments (particularly multiplex assays), the disclosed kits and/or reaction volumes can include TaqMan Fast Virus 1-Step Master Mix (sold by Thermo Fisher Scientific under Catalog No. 44444432), or alternatively, TaqPath 1-Step RT-qPCR Master Mix, CG (sold by Thermo Fisher Scientific under Catalog No. A15299). In other embodiments the master mix is TaqPath 1 Step Multiplex Master Mix (No ROX) (sold by Thermo Fisher Scientific under Catalog Nos. A48111 and A28521) or similar master mix known in the art. In some embodiments, the kit includes primers, probes, and master mix sufficient to constitute a reaction mixture supporting multiplex amplification of one or more SARS-CoV-2 regions encoding the N protein, the S protein and/or ORF1ab protein in a single reaction volume.

[0092] In some embodiments, the kit includes at least one qPCR assay (including forward primer, reverse primer, probe, and optionally a master mix or components thereof) configured to amplify a region of the gene encoding the ORF1ab protein. In some embodiments, the kit includes at least one qPCR assay (including forward primer, reverse primer, probe, and optionally a master mix or components thereof) configured to amplify a region of the gene encoding the N protein. In some embodiments, the kit includes at least one qPCR assay (including forward primer, reverse primer, probe, and optionally a master mix or components thereof) configured to amplify a region of the gene encoding the S protein. In some embodiments, the kit includes at least one qPCR assay (including forward primer, reverse primer and optionally a probe) configured to amplify a region of the gene encoding the N protein, at least one qPCR assay (including forward primer, reverse primer, probe, and optionally a master mix or components thereof) configured to amplify a region of the gene encoding the S protein, and/or at least one qPCR assay (including forward primer, reverse primer, probe, and optionally a master mix or components thereof) configured to amplify a region of the gene encoding the ORF1ab protein.

[0093] In some embodiments, the primers and/or probes associated with SEQ ID NO:4 – SEQ ID NO:2533 and/or Table 2 may further comprise a fluorescent or other detectable label and/or a

quencher or minor groove binder, such as those described above. As a non-limiting example, said primers and/or probes can be associated with FAM, ABY, VIC, or JUN as detectable labels and QSY as a quencher. As such, it should be appreciated that the primers and probes of SEQ ID NO:4 – SEQ ID NO:2533 and/or Table 2 may be included with disclosed kits, arrays, etc. for use in either singleplex or multiplex assay formats.

[0094] In some embodiments of multiplex assay formats described herein, one or more various SARS-CoV-2 genomic regions are detected, including assays for detecting the coding regions of ORF1ab (*e.g.,* FAM-labeled), N Protein (*e.g.,* VIC-labeled), and/or S Protein (*e.g.,* ABY-labeled). As described above, one or more labelled primers may be used, in addition to or as an alternative to labelled probes, for detecting one or more target nucleic acids. Thus, in some embodiments, no probes are utilized.

[0095] In some embodiments of multiplex assay formats described herein, various SARS-CoV-2 genomic regions are detected, including assays for the coding regions of N Protein and S Protein (*e.g.,* both FAM-labeled), optionally combined with assays for Flu A (*e.g.,* VIC-labeled) and/or Flu B (e.g., ABY-labeled). In some embodiments of multiplex assay formats described herein, various SARS-CoV-2 genomic regions are detected, including assays for detecting the coding regions of N Protein and S Protein (*e.g.,* VIC-labeled), optionally combined with assays for Flu A and/or B (*e.g.,* both FAM-labeled) and/or for RSV Type A and/or Type B (*e.g.,* both ABY-labeled). Optionally, in some embodiments, a control (*e.g.,* JUN-labeled), such as bacteriophage MS2 or RNase P control, is included in the kit, array, etc. comprising the multiplex assay. It should be appreciated that although particular examples are provided above indicating a given fluorophore associated with detection of a given viral sequence, the primers and/or probes can be modified to include a functionally similar fluorophore described herein or as otherwise known in the art. Further, quenchers, such as QSY, can be included in any of the foregoing examples, and the detectable label and/or quencher can be selected based on the singleplex or multiplex requirements of the given qPCR assay in accordance with the constraints and considerations discussed above or otherwise understood by those having skill in the art.

[0096] In some embodiments, at least one of the qPCR assays targets a sequence within a SARS-CoV-2 gene selected from the group consisting of: the N protein, the ORF1ab protein, and the S protein. In some embodiments, the reaction volume further includes a second qPCR assay targeting a different gene of the aforementioned group. In some embodiments, the reaction volume further includes a third qPCR assay that targets the remaining third gene from the aforementioned group, such that when the reaction volume is subjected to amplification conditions, and if the sample includes SARS-CoV-2 genomic RNA, at least one amplicon is produced from genetic

sequence encoding the S protein, at least one amplicon from genetic sequence encoding the N protein and at least one amplicon from the genetic sequence encoding the ORF1ab protein.

[0097] In a further multiplex format, at least two qPCR assays are combined into a single reaction volume that includes a nucleic acid sample obtained or derived from body tissue, as described herein, and at least one exogenous positive control nucleic acid sequence. The exogenous positive control sequence may comprise an MS2 phage sequence/gene or other nucleic acid sequence, preferably RNA sequence. Additionally, or alternatively, the multiplex qPCR assays include an endogenous positive control, such as human RNase P. In some embodiments, the primer and/or probe sequences described by the United States Centers for Disease Control and Prevention (CDC) may be utilized (https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html). For example, assays described herein may include one or more primers and/or probes shown in Table 2.

Table 2: CDC 2019-Novel Coronavirus (2019-nCoV) Real-time RT-PCR Primers and Probes

Name/Description	**SEQ ID NO**	**Labels[1,2]**	**Oligonucleotide Sequence (5' > 3')**	**Final Conc.***
2019-nCoV_N1 Forward Primer	SEQ ID NO:2544	None	GAC CCC AAA ATC AGC GAA AT	500 nM
2019-nCoV_N1 Reverse Primer	SEQ ID NO:2545	None	TCT GGT TAC TGC CAG TTG AAT CTG	500 nM
2019-nCoV_N1 Probe	SEQ ID NO:2546	FAM, BHQ-1	FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1	125 nM
2019-nCoV_N1 Probe	SEQ ID NO:2547	FAM, ZEN, 3IABkFQ	FAM-ACC CCG CAT /ZEN/ TAC GTT TGG TGG ACC-3IABkFQ	125 nM
2019-nCoV_N2 Forward Primer	SEQ ID NO:2548	None	TTA CAA ACA TTG GCC GCA AA	500 nM
2019-nCoV_N2 Reverse Primer	SEQ ID NO:2549	None	GCG CGA CAT TCC GAA GAA	500 nM
2019-nCoV_N2 Probe	SEQ ID NO:2550	FAM, BHQ-1	FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1	125 nM
2019-nCoV_N2 Probe	SEQ ID NO:2551	FAM, ZEN, 3IABkFQ	FAM-ACA ATT TGC /ZEN/ CCC CAG CGC TTC AG-3IABkF	125 nM
RNase P Forward Primer	SEQ ID NO:2552	None	AGA TTT GGA CCT GCG AGC G	500 nM
RNase P Reverse Primer	SEQ ID NO:2553	None	GAG CGG CTG TCT CCA CAA GT	500 nM
RNase P Probe	SEQ ID NO:2554	FAM, BHQ-1	FAM – TTC TGA CCT GAA GGC TCT GCG CG – BHQ-1	125 nM

Name/Description	SEQ ID NO	Labels[1,2]	Oligonucleotide Sequence (5' > 3')	Final Conc.*
RNase P Probe	SEQ ID NO:2555	FAM, ZEN, 3IABkFQ	FAM-TTC TGA CCT /ZEN/ GAA GGC TCT GCG CG-3IABkFQ	125nM
RNase P Probe	SEQ ID NO:2556	VIC, QSY	VIC-TTC TGA CCT GAA GGC TCT GCG CG-QSY	125nM

[1] – The labels shown are exemplary to some embodiments of the compositions, reactions mixtures, kits, or methods described herein and are in no way meant to limit other possible labels, including various fluorophores and quenchers, contemplated for use in the primers and probes described herein.

[2] – TaqMan® probes are labeled at the 5′-end with the reporter molecule 6-carboxyfluorescein (FAM) and with the quencher, Black Hole Quencher 1 (BHQ-1) (Biosearch Technologies, Inc., Novato, CA) at the 3′-end. TaqMan® probes can also be labeled at the 5′-end with the reporter molecule 6-carboxyfluorescein (FAM) and with a double quencher, ZEN™ Internal Quencher positioned between the ninth (9th) and tenth (10th) nucleotide base in the oligonucleotide sequence and Iowa Black® FQ (3IABkFQ) located at the 3'-end (Integrated DNA Technologies, Coralville, IA).

* – The final concentrations shown are exemplary to some embodiments of the compositions, reactions mixtures, kits, or methods described herein and are in no way meant to limit other possible concentrations or concentration ranges for use in the compositions, reactions mixtures, kits, or methods contemplated herein.

[0098] In some embodiments, kits are provided that include reagents for a multiplex qPCR assay targeting a SARS-CoV-2 sequence selected from the coding regions of the ORF1ab protein, the N protein, and/or the S protein. In some embodiments, the kits additionally include reagents for a second qPCR assay targeting a different, SARS-CoV-2 sequence selected from the coding regions of the ORF1ab protein, the N protein, and the S protein. In some embodiments, the kits additionally include reagents for a third qPCR assay targeting the third SARS-CoV-2 sequence selected form the coding regions associated with the ORF1ab protein, the N protein, and the S protein, such that when a reaction volume containing reagents from the first, second, and third qPCR assays is subjected to amplification conditions—and if the nucleic acid sample being tested includes SARS-CoV-2 genomic RNA (or cDNA reverse transcribed therefrom)—at least one amplicon is produced from the coding region associated with the ORF1ab protein, at least one amplicon is produced from the coding region associated with the S protein, and at least one amplicon is produced from the coding region associated with the N protein (*e.g.,* using a first forward primer selected from SEQ ID NO:4, SEQ ID NO:34, and SEQ ID NO:160, a second forward primer selected from SEQ ID NO:5 and SEQ ID NO:100, and a third forward primer selected from SEQ ID NO:211 and SEQ ID NO:248; using a first reverse primer selected from SEQ ID NO:320, SEQ ID NO:423, and SEQ ID NO:468, a second reverse primer selected from SEQ ID NO:337 and SEQ ID NO:441, and a third reverse primer selected from SEQ ID NO:487

SEQ ID NO:501, and 510; and using complementary probes selected from SEQ ID NO:520 – SEQ ID NO:1295 and/or SEQ ID NO:1466 – SEQ ID NO:2359; preferably one or more probes selected from SEQ ID NO: 821 – 1054, SEQ ID NO: 1853 – 2027, SEQ ID NO: 2028 – 2220, and/or SEQ ID NO: 2221 – 2359; and more preferably one or more probes selected from SEQ ID NO:565, SEQ ID NO:599, SEQ ID NO:833, SEQ ID NO:864, SEQ ID NO:930, SEQ ID NO:971, SEQ ID NO:1049, SEQ ID NO:1106, SEQ ID NO:1160, SEQ ID NO:1203, SEQ ID NO:1866, SEQ ID NO:1891, SEQ ID NO:1962, SEQ ID NO:2031, SEQ ID NO:2188, SEQ ID NO:2203, SEQ ID NO:2216, SEQ ID NO:2248, SEQ ID NO:2291, and/or SEQ ID NO:2345). In some embodiments, the first forward primer is SEQ ID NO: 160. In some embodiments, the second forward primer is SEQ ID NO: 100. In some embodiments, the third forward primer is SEQ ID NO: 211. In some embodiments, the first reverse primer is SEQ ID NO: 468. In some embodiments, the second reverse primer is SEQ ID NO: 337. In some embodiments, the third reverse primer is SEQ ID NO: 501 and/or 510. In some embodiments, the complementary probes are SEQ ID NOs: 1049, 864 and/or 833. In some embodiments the first forward primer is SEQ ID NO: 160, the second forward primer is SEQ ID NO: 100, the third forward primer is SEQ ID NO: 211, the first reverse primer is SEQ ID NO: 468, the second reverse primer is SEQ ID NO: 337, the third reverse primer is SEQ ID NO: 501 and/or 510, and the complementary probes are SEQ ID NOs 1049, 864 and 833, respectively.

[0099] The primer and probe sequences described herein need not have 100% homology to their targets to be effective, though in some embodiments, homology is substantially 100%. In some embodiments, one or more of the disclosed primer and/or probe sequences have a homology to their respective target of about 50%, about 60%, about 70%, about 80%, about 85%, about 90%, about 95%, about 97%, about 98%, about 99%, or up to substantially 100%. Some combinations of primers and/or probes may include primers and/or probes each with different homologies to their respective targets, and the homologies may be, for example, within a range with endpoints defined by any two of the foregoing values.

[0100] In some embodiments, the multiplex qPCR kits disclosed herein additionally include reagents for a fourth qPCR assay targeting an exogenous/endogenous positive control sequence, such that when the multiplex reaction volume is subjected to amplification conditions—and if the sample includes SARS-CoV-2 genomic RNA and subject-derived nucleic acid or exogenously added template nucleic acid—at least one amplicon is produced from the coding region associated with the ORF1ab protein, at least one amplicon is produced from the coding region associated with the S protein, at least one amplicon is produced from the coding region associated with the N protein, as above, and at least one amplicon is produced from the exogenous/endogenous positive

control sequence. If the positive control sequence is an endogenously-derived control, such as RNase P, the presence of subject-derived nucleic acid (*e.g.,* genomic DNA coding for RNase P, RNase P RNA, and/or reverse transcribed RNase P transcript), can be used as the template for an RNase P qPCR assay. Exemplary primers and probes for such an RNase P qPCR positive control can include SEQ ID NO:2552 – SEQ ID NO:2556, although those having skill in the art should appreciate that other RNase-P-specific primers and/or probes could be used. If the positive control sequence is an exogenously-derived control, such as a component of the MS2 bacteriophage, a known or predetermined concentration of template nucleic acid is added to the reaction volume to serve as the requisite template for an MS2 qPCR assay. Exemplary primers and probes for an exogenous MS2 qPCR positive control can include SEQ ID NO:256, SEQ ID NO:509, and SEQ ID NO:1300, although those having skill in the art should appreciate that other MS2-specific primers and/or probes could be used.

[0101] In some embodiments, the singleplex and multiplex assays can include synthetic DNA controls and/or genomic RNA controls for the pathogenic targets being tested, clinical isolate research samples, and/or organism samples. The compositions and kits can also include a control DNA construct comprising a plasmid carrying the target sequences of the N protein, the S protein and the ORF1ab protein. The control plasmid can additionally include an RNase P coding sequence for use as a positive control and/or a Xeno cassette for monitoring sample contamination. In some embodiments, the synthetic control construct is provided in a separate kit with the same primers and probes included within a complementary kit used to detect SARS-CoV-2 viral nucleic acid from a biological sample. These two kits can be manufactured, in some embodiments, at different locations to prevent any possible cross-contamination of the control plasmid within the detection kits (which would result in false positive sample results).

[0102] In some embodiments, the multiplex RT-qPCR kits disclosed herein additionally include reagents for a fifth qPCR assay that targets two separate (exogenous/endogenous) positive control sequences, such that when the reaction volume is subjected to amplification conditions—and if the sample includes SARS-CoV-2 genomic RNA and subject-derived nucleic acid or exogenously added template nucleic acid—at least one amplicon is produced from the coding region associated with the ORF1ab protein, at least one amplicon is produced from the coding region associated with the S protein, at least one amplicon is produced from the coding region associated with the N protein, as above, and at least two amplicons are produced from the exogenous and/or endogenous positive control sequence(s) (*e.g.,* using primers and probes for RNase P and/or MS2 described above, or other selective and specific primers and probes that can serve as a positive control in the qPCR assays described herein).

[0103] In some embodiments, the disclosed primers and probes (including those listed in SEQ ID NO:4 – SEQ ID NO:2533) are used in a multiplex assay format that targets at least two different loci in the SARS-CoV-2 genome, namely: the gene encoding ORF1ab, the gene encoding the S protein, and/or the gene encoding the N protein. In some embodiments, the disclosed primers and probes are used in a multiplex assay format that targets at least three loci in the SARS-CoV-2 genome, namely: the gene encoding ORF1ab, the gene encoding the S protein, and the gene encoding the N protein. A single qPCR reaction volume includes multiple assays for each of these target genes to ensure redundancy and specific identification of the target virus.

[0104] For example, a multiplex assay of the present disclosure includes a 4-plex assay where three of the four dye channels are each dedicated to the identification of a different SARS-CoV-2 coding region, particularly portions of the coding regions associated with the N protein, the S protein, or ORF1ab that allow for the specific and selective identification of SARS-CoV-2. The fourth dye channel can be used for the identification of a positive control (*e.g.,* an exogenously added control like MS2 or an endogenously present control like RNase P). That is, multiplex assays of the present disclosure can include probes having sequence specificity for coding regions of the N protein, S protein, or ORF1ab coupled to a detectable label and/or quencher as proved herein. As a non-specific example, a multiplex assay can include a first probe targeting a SARS-CoV-2-specific N protein coding sequence and having a VIC dye associated therewith. A second probe of the multiplex assay can target a SARS-CoV-2-specific S protein coding sequence and have an ABY dye associated therewith. A third probe of the multiplex assay can target a SARS-CoV-2-specific ORF1ab coding sequence and have a FAM dye associated therewith. A fourth probe of the multiplex assay can be specific for a positive control sequence and have a JUN dye associated therewith. One having skill in the art will recognize that the foregoing dyes can be interchanged or exchanged for another detectable label known in the art.

[0105] In some embodiments, a 4-plex assay includes at least two SARS-CoV-2-specific probes sharing the same dye channel. Second and third dye channels of the 4-plex assay can be associated with Flu A- and Flu B-specific probes, respectively, and the fourth dye channel can be associated with RSV A and/or RSV B specific probes or a positive control probe. Alternatively, a second dye channel can be shared by Flu A and Flu B specific probes, a third channel can be associated with RSV A and/or RSV B specific probes, and a fourth dye channel can be associated with a positive control probe. Alternatively, the third dye channel can be associated with a Flu A-specific probe, and the fourth channel can be associated with a Flu B-specific probe.

[0106] In some embodiments, a 4-plex assay includes at least two SARS-CoV-2-specific probes sharing the same dye channel. Second and third dye channels of the 4-plex assay can be

associated with RSV A-specific and RSV B-specific probes, respectively, and the fourth dye channel can be associated with Flu A- and/or Flu B-specific probes or a positive control probe. Alternatively, a second dye channel can be shared by RSV A and RSV B specific probes, a third channel can be associated with Flu A- and/or Flu B-specific probes, and a fourth dye channel can be associated with a positive control probe. Alternatively, the third dye channel can be associated with an RSV A-specific probe, and the fourth channel can be associated with an RSV B-specific probe.

[0107] The disclosed primers and probes can be included within additional and/or alternative multiplex assays. For example, a 5-plex assay can include at least two SARS-CoV-2-specific probes (e.g., for N protein, S protein, and/or ORF1ab amplicons). The probes can be associated with different dye channels or can share the same dye channel. In some embodiments, detection between CoV-2 N Protein and S Protein genomic sequences is not distinguished. In some embodiments, detection between CoV-2 N Protein genomic sequence and S Protein genomic sequence is distinguished. Additional, second, third, and/or fourth, dye channels can be associated with, for example, Flu A-specific and/or Flu B-specific probes and/or RSV A-specific and/or RSV B-specific probes. A positive control probe can additionally be included in some embodiments. As a particular, non-limiting example, regions within the genes encoding SARS-CoV-2 N protein, S protein, and/or ORF1ab may be detected using a single dye channel (e.g., VIC) while combined assays for Influenza Type A and/or B and/or for RSV Type A and/or B are simultaneously detected in the same reaction using one or more different dye channels (e.g., FAM, ABY and/or JUN). In one embodiment, at least two SARS-CoV-2-specific detectable primers and/or probes can be associated with a single dye channel, such as by labeling with a VIC dye, and optionally, a Flu A-specific detectable primer and/or probe can be associated with second dye channel, such as by labeling with an ABY dye and/or a Flu B-specific detectable primer and/or probe can be associated with a third dye channel, such as by labeling with a FAM dye. In another embodiment, assays for SARS-CoV-2 and/or Influenza Type A and/or Type B can be further combined with RSV A and RSV B detectable primers and/or probes to include detection through yet another dye channel, such as through respective association a JUN dye. Optionally, a fifth channel can be reserved for an MS2 or RNase P positive control primers and/or probes associated with an ALEXA (AF) dye.

Compositions, Kits, and Methods for Detection of Multiple Respiratory Tract Pathogens

[0108] The compositions, kits, and methods for detecting SARS-CoV-2, described above and elsewhere herein, can form the basis of an assay for detecting multiple respiratory tract pathogens. For example, the qPCR assays described above (whether singleplex or multiplex) can be included

as a component of a panel of qPCR assays or as a multiplex assay for detecting a plurality of pathogens from a subject sample.

[0109] In some embodiments, a panel of qPCR assays includes one or more assays for detecting SARS-CoV-2 in addition to one or more assays for detecting Flu A and/or Flu B viruses. In some embodiments, the panel of qPCR assays includes one or more assays for SARS-CoV-2 in addition to one or more assays for RSV A and/or RSV B. In some embodiments, the panel of qPCR assays includes one or more assays for SARS-CoV-2 in addition to one or more assays for Flu A and/or Flu B viruses, and one or more assays for RSV A and/or RSV B. In some embodiments, the panel of qPCR assays includes at least two assays for SARS-CoV-2 in addition to at least two assays for Flu A and/or Flu B viruses, and at least two assays for RSV A and/or RSV B.

[0110] It should be appreciated that the panel of qPCR assays can be performed in a singleplex format, allowing the probes for each qPCR target to be shared between the various assays. Alternatively, the qPCR assays can be performed in a multiplex format with each component of the panel being associated with a different probe whose emission spectra do not substantially overlap (*i.e.,* the emission spectra of each probe is uniquely identifiable from the other probes).

[0111] In some embodiments, the panel of qPCR assays includes one or more assays for SARS-CoV-2 in addition to one or more assays for a control sequence. In some embodiments, the control sequence is an RNase P sequence and/or a MS2 Phage sequence. In some embodiments, the panel of qPCR assays includes one or more assays comprising any of the forward primers, reverse primers, and probe sequences listed in SEQ ID NO:4 – SEQ ID NO:2533. It should be appreciated that qPCR and RT-qPCR methods are known to those having skill in the art. Nevertheless, particular embodiments are provided in the Examples illustrating use of qPCR assays for detecting SARS-CoV-2, Flu A and/or Flu B, and/or RSV A, and/or RSV B targets.

[0112] In some embodiments, the panel of qPCR assays includes four or more assays comprising any of the forward primers, reverse primers, and probe sequences listed in SEQ ID NO:4 – SEQ ID NO:2533. In some embodiments, the panel of qPCR assays includes six or more assays comprising any of the forward primers, reverse primers and probes sequences listed in SEQ ID NO:4 – SEQ ID NO:2533. Each qPCR assay can include a forward primer and a reverse primer. Optionally, the assay further includes a probe, which can be a hydrolysis probe. In some embodiments, each qPCR assay includes at least one forward primer, at least one reverse primer, and at least one probe selected from any of those listed in SEQ ID NO:4 – SEQ ID NO:257, SEQ ID NO:267 – SEQ ID NO:510, and SEQ ID NO:520 – SEQ ID NO:2533, respectively.

[0113] In some embodiments, the panel of qPCR assays includes at least one qPCR assay for detecting SARS-CoV-2, plus at least one qPCR assay for detecting at least one assay for a

respiratory microorganism listed in Table 3A, below. In some embodiments, the panel of qPCR assays includes at least one qPCR assay for detecting SARS-CoV-2, plus at least one qPCR assay for detecting each of the microorganisms listed in Table 3A. In some embodiments, the assay detects two or more (*e.g.,* 2, 3, 4, 5, 6, etc.) of the targets of Table 3A. In some embodiments, the assay detects at least two targets within the SARS-CoV-2 genome as well an internal positive control (see, e.g., Table 3B), such as human RNase P. In some embodiments, the assay simultaneously detects every target of Table 3A in addition to one or more positive control, such as for RNase P and/or 18S rRNA and/or an exogenous control like bacteriophage MS2 (see, e.g., Table 3A). In some embodiments, the panel of assays is formatted as an open array card. In other embodiments, the panel of assays is formatted as an open array plate. In some embodiments, the panel of assays are used in a plurality of singleplex assays. In some other embodiments, the panel of assays are used in one or more multiplex assays. In some embodiments, the panel of assays includes pooled assays. In some embodiments, the panel of assays includes dried down and/or lyophilized assays.

Table 3A. List of targets and assays for respiratory tract microbiota (RTM)

Target organism	**Assay name**	**Nucleic acid**	**Assay ID (Thermo Fisher Scientific)**
Bacteria			
Bordetella bronchiseptica / parapertussis / pertussis	Bordetella	DNA	Ba06439624_s1
Bordetella pertussis	*B. pertussis*	DNA	Ba06439623_s1
Chlamydophila pneumoniae	*C. pneumoniae*	DNA	Ba06439616_s1
Haemophilus influenzae	*H. influenzae*	DNA	Ba06439625_s1
Klebsiella pneumoniae	*K. pneumoniae*	DNA	Ba04932083_s1
Legionella pneumophila	*L. pneumophila*	DNA	Ba06439617_s1
Mycoplasma pneumoniae	*M. pneumoniae*	DNA	Ba06439620_s1
Staphylococcus aureus	*S. aureus*	DNA	Ba04646259_s1
Streptococcus pneumoniae	*S. pneumoniae*	DNA	Ba06439619_s1
Bordetella holmesii	*B. holmesii*	DNA	Ba06439621_s1
Coxiella burnetii	*C. burnetii*	DNA	Ba06439618_s1
Moraxella catarrhalis	*M. catarrhalis*	DNA	Ba06439622_s1
Virus			
Adenovirus	AdV_1of2	DNA	Vi99990001_po
Adenovirus	AdV_2of2	DNA	Vi99990002_po
Human Bocavirus	HBoV	DNA	Vi99990003_po
Human Coronavirus 229E	CoV_229E	RNA	Vi06439671_s1

Human Coronavirus HKU1	CoV_HKU1	RNA	Vi06439674_s1
Human Coronavirus NL63	CoV_NL63	RNA	Vi06439673_s1
Human Coronavirus OC43	CoV_OC43	RNA	Vi06439646_s1
Human Enterovirus (pan assay)	EV_pan	RNA	Vi06439631_s1
Human Enterovirus D68	EV_D68	RNA	Vi06439669_s1
Human Metapneumovirus (hMPV)	hMPV	RNA	Vi99990004_po
Human Parainfluenza virus 1	hPIV1	RNA	Vi06439642_s1
Human Parainfluenza virus 2	hPIV2	RNA	Vi06439672_s1
Human Parainfluenza virus 3	hPIV3	RNA	Vi06439670_s1
Human Parainfluenza virus 4	hPIV4	RNA	Vi99990005_po
Human Respiratory Syncytial Virus A (RSVA)	RSVA	RNA	Vi99990014_po
Human Respiratory Syncytial Virus B (RSVB)	RSVB	RNA	Vi99990015_po
Human Rhinovirus 1/2	RV_1of2	RNA	Vi99990016_po
Human Rhinovirus 2/2	RV_2of2	RNA	Vi99990017_po
Human herpesvirus 3 (HHV3 – Varicella zoster Virus)	HHV3	DNA	Vi06439647_s1
Human herpesvirus 4 (HHV4 – Epstein-Barr Virus)	HHV4	DNA	Vi06439675_s1
Human herpesvirus 5 (HHV5 – Cytomegalovirus)	HHV5	DNA	Vi06439643_s1
Human herpesvirus 6 (HHV6)	HHV6	DNA	Vi06439627_s1
Influenza A	Flu_A_pan	RNA	Vi99990011_po
Influenza A/H1-2009	Flu_A_H1	RNA	Vi99990009_po
Influenza A/H3	Flu_A_H3	RNA	Vi99990010_po
Influenza B	Flu_B_pan	RNA	Vi99990012_po
Human Parechovirus	HPeV	RNA	Vi99990006_po
Measles virus	Measles	RNA	Vi99990013_po
Middle East Respiratory Syndrome coronavirus (MERS)	MERS_CoV	RNA	Vi06439644_s1
Mumps virus	Mumps	RNA	Vi06439657_s1
Severe Acute Respiratory Syndrome coronavirus (SARS)	SARS_CoV	RNA	Vi06439634_s1
Fungus			
Pneumocystis jirovecii	*P. jirovecii*	DNA	Fn06439626_s1

Table 3B: Assays for respiratory tract microbiota controls

Control name	**Assay name**	**Nucleic acid**	**Assay ID (Thermo Fisher Scientific)**
TaqMan® Universal Extraction Control Organism (*B. atrophaeus*)	*B. atrophaeus*	DNA	Ba06596576_s1
TaqMan® Universal RNA Spike In/Reverse Transcription (Xeno) Control	Xeno	RNA	Ac00010014_a1
Human RNase P RPPH1 gene	RPPH1	DNA	Hs04930436_g1

[0114] In some embodiments, a panel of different qPCR assays can be used to test for multiple strains or types of pathogens in addition to SARS-CoV-2, including, but not limited to, other viral pathogens, such as Flu A and/or Flu B, and RSV Type A and/or Type B, bacterial pathogens, and/or fungal pathogens. In some embodiments, the panel of qPCR assays can be used simultaneously to test a single subject sample or a pooled sample comprising multiple subject samples, with each assay run in parallel in an array format. Optionally, different qPCR assays specific for each of the target nucleic acids can be plated into individual wells of a single array or multi-well plate, similar to, for example, a TaqMan Array Card (*e.g.,* sold by Thermo Fisher Scientific under Catalog Nos. 4346800 and 4342265) or a MicroAmp multi-well reaction plate (*e.g.*, sold by Thermo Fisher Scientific under Catalog Nos. 4346906, 4366932, 4306737, 4326659 and N8010560). Optionally, the different qPCR assays present in different wells of an array or plate can be dried or freeze-dried *in situ* and the array or plate can be stored or shipped prior to use. In some embodiments the array-formatted assays can be run as a singleplex or as a multiplex assay.

[0115] An exemplary array card disclosed herein can include some or all of the assays present in the TaqMan Array Respiratory Tract Microbiota Comprehensive Card (sold by Thermo Fisher Scientific under Catalog No. A41238), particularly those directed to identifying viral, bacterial, or fungal microbes from a sample, along with one or more assays for detecting SARS-CoV-2, as disclosed herein, present in at least one well of the array. The SARS-CoV-2 detection assays can include at least one primer or probe selected from SEQ ID NO:4 – SEQ ID NO:2533.

[0116] In some embodiments, the panel includes assays for other circulating coronavirus strains, including but not limited to the 229E, KHU1, NL63, and OC43 coronaviruses. The panel/array card can include any number of individual assays, but in some embodiments, the panel/array card includes 48 separate assays, at least one of which is a control assay (*e.g.,* RNase P, 18S rRNA, or the like). In some embodiments, the disclosed methods include using the panel to profile respiratory microorganisms present in a sample taken from an organism (e.g., human) and determining the profile of respiratory microbiota present in the organism's sample. Optionally, the

disclosed methods can include diagnosing an infection present in an organism (e.g., human) from which a sample is taken.

[0117] In any of the foregoing embodiments, it should be appreciated that the compositions, kits, and methods for detecting viral nucleic acid can be implemented at or be included within a "point-of-service" (POS) system. Additionally, or alternatively, samples may be collected and/or analyzed at a "point-of-care" (POC) location. In some embodiments, analysis at a POC location typically does not require specialized equipment and has rapid and easy-to-read visual results. In some embodiments, analysis can be performed in the field, in a home setting, and/or by a lay person not having specialized skills. In certain embodiments, for example, the analysis of a small-volume clinical sample may be completed using a POS system in a short period of time (e.g., within hours or minutes).

[0118] Optionally, a "point of service" (POS) or a "point of service system," is performed at a location, using a system at that location, which is capable of providing a service (e.g. testing, monitoring, treatment, diagnosis, guidance, sample collection, verification of identity (ID verification), and other services) at or near the site or location of the subject. A service may be a medical service or may be a non-medical service. In some situations, a POS system provides a service at a predetermined location, such as a subject's home, school, or work, or at a grocery store, a drug store, a community center, a clinic, a doctor's office, a hospital, an outdoor triage tent, a makeshift hospital, a border check point, etc. A POS system can include one or more point of service devices, such as a portable virus/pathogen detector. In some embodiments, a POS system is a point of care system. In some embodiments, the POS system is suitable for use by non-specialized workers or personnel, such as nurses, police officers, civilian volunteers, or the subject herself.

[0119] In certain embodiments, the assays described herein can be performed at a "point of care" (POC), e.g., a location at which medical-related care (e.g. treatment, testing, monitoring, diagnosis, counseling, etc.) is provided. A POC may be, e.g. at a subject's home, work, or school, or at a grocery store, a community center, a drug store, a doctor's office, a clinic, a hospital, an outdoor triage tent, a makeshift hospital, a border check point, etc. A POC system is a system which may aid in, or may be used in, providing such medical-related care, and may be located at or near the site or location of the subject or the subject's health care provider (e.g. subject's home, work, or school, or at a grocery store, a community center, a drug store, a doctor's office, a clinic, a hospital, etc.).

[0120] In embodiments, such a system is a point-of-service system (POS system), wherein a POS system is located at a point of service location. In embodiments, a POS system is located at a

point of service location and is configured to accept a clinical sample obtained from a subject at the POS location. In embodiments, a POS system is located at a point of service location and is configured to accept a clinical sample obtained from a subject at the POS location, and is further configured to analyze the clinical sample at the POS location. In embodiments, the clinical sample is a small volume clinical sample. In embodiments, the clinical sample is analyzed in a short period of time. In embodiments, the short period of time is determined with respect to the time at which sample analysis began. In embodiments, the short period of time is determined with respect to the time at which the sample was inserted into a device for the analysis of the sample. In embodiments, the short period of time is determined with respect to the time at which the sample was obtained from the subject.

[0121] In some embodiments, a POS can include the amplification-based methods, compositions and kits disclosed herein, including any of the described assays and/or assay panels. Such assays are contemplated for use with both thermal cycling amplification workflows and protocols, such as in PCR, as well as isothermal amplification workflows and protocols, such as in LAMP.

[0122] In some embodiments, a POS or a POC comprises self-collection of a biological sample, such as a nasal swab or a saliva sample. In some embodiments, the self-collection may comprise the use of a self-collection kit and/or device, such as a swab or a tube. In some embodiments, the self-collection kit comprises instructions for use, including collection instructions, sample preparation or storage instructions, and/or shipping instructions. For example, the self-collection kit and/or device may be used by an individual, such as lay person, not having specialized skills or medical expertise. In some embodiments, self-collection may be performed by the subject themselves or by any another individual in proximity to the subject, such as but not limited to a parent, a care giver, a teacher, a friend, or other family member.

[0123] The POS/POC implementations can beneficially provide a convenient way to monitor and/or detect individuals who may be infected by any of the viruses sought by the disclosed kits and methods. This includes, for example, screening for asymptomatic individuals (*e.g.,* those individuals who are infected with SARS-CoV-2 and who may be shedding infectious virions prior to the presentation of the hallmark symptoms associated with respiratory tract infections (*e.g.,* fever, coughing, malaise). Once detected, the infected individuals can be quarantined to prevent further spread of the infection. Additionally, or alternatively, the individual can be provided appropriate medical care, which in some embodiments can be initiated directly at the POC facility and/or following notification of a healthcare professional by the POS system/POC facility.

[0124] The disclosed kits and methods for detecting viral sequences beneficially provides companies and/or venues that are decentralized from hospitals and/or traditional clinical laboratory services with the ability to quickly screen samples on site and to take action where appropriate and with only those individuals where action should be taken. For example, spectators of a sporting event may arrive at the corresponding venue in advance of the event where they can be screened for SARS-CoV-2 (in addition to any other disclosed virus). Those spectators who test negative may then enter the venue and may not be required to practice social distancing or to wear personal protective equipment, such as wearing a face covering. On the other hand, those spectators who test positive can be rejected entry and directed to a healthcare professional, thereby limiting the exposure and/or spread of SARS-CoV-2 (or other virus detected by the disclosed kits and methods disclosed herein) at the venue.

[0125] Because the disclosed kits and methods enable screening of individuals at locations that are decentralized from hospitals and/or traditional clinical laboratory services, the disclosed kits and/or methods can additionally be used to collect accurate epidemiological data from a population more efficiently and more quickly. Such data can be used to identify hot spots within a community and/or behaviors or cohorts that perpetuate the spread of such viral pathogens, which can enable directed or more effective solutions.

[0126] When conducting screenings, as discussed above, the samples from individuals who report to be healthy (*i.e.,* asymptomatic for the hallmarks of SARS-CoV-2, or other respiratory tract, infection) can be combined into a single pooled sample. Because most of the samples are expected to be negative, such methods can beneficially enable the screening of large populations quickly and efficiently, though it may require a minority of people to be retested to determine which sample(s) in a given pooled sample is responsible for the pooled sample testing positive for the presence of the target nucleic acid (*e.g.,* SARS-CoV-2). Less assay resources and/or instruments would be required to process each sample separately and/or the same amount of assay resources and/or instruments can be used to screen a larger number of samples.

[0127] That is, in some embodiments, a sample is obtained from multiple organisms (*e.g.,* a plurality of subjects or patients) and the multiple samples are pooled together to make a single pooled sample for testing. A sample may be obtained from at least two different organisms or individuals for pooling together to form a single pooled sample for testing. In some embodiments, a sample may be obtained from between 2–10 different organisms or individuals and pooled together to form a single sample for testing. In some embodiments, a sample may be obtained from 2, 3, 4, 5, 6, 7, 8, 9, or 10 different organisms or individuals for pooling together to form a single sample for testing. In some embodiments, a sample may be obtained from up to and including 5

different organisms or individuals for pooling together to form a single sample for testing. For example, a sample used for testing, according to the methods and compositions described herein, may comprise a multiplicity of samples obtained from different organisms or individuals (e.g. 2, 3, 4, 5 different individuals) which are combined together to form a single samples used for subsequent detection of a pathogen such as SARS-CoV-2.

[0128] Regardless of whether the samples are pooled or evaluated individually, the inventors have unexpectedly discovered that mixing RT-qPCR reaction volumes can play a critical role in preventing optical mixing and/or the false classification of samples. Accordingly, a step of mixing (*e.g.,* vortexing) the reaction volume prior to RT-qPCR is included in preferred methods for detecting viral sequences disclosed herein. This can include, for example, vortexing the reaction volume (*e.g.,* single tube, array, and/or plate-based). In some embodiments, the reaction volume is vortexed for at least 5 seconds, preferably at least 10 seconds. In some embodiments, the reaction volume is vortexed for about one minute or longer, preferably less than about one minute, or more preferably less than about 30 seconds. In some embodiments, the reaction volume is vortexed for a period of time between 5 seconds and about one minute, preferably between 10 – 30 seconds.

Abbreviated list of defined terms

[0129] To assist in understanding the scope and content of the foregoing and forthcoming written description and appended claims, a select few terms are defined directly below.

[0130] The SARS-CoV-2 virus, also known as 2019-nCoV, is associated with the human respiratory disease COVID-19. The virus isolated from early cases of COVID-19 was provisionally named 2019-nCoV, and the Coronavirus Study Group of the International Committee on Taxonomy of Viruses subsequently designated 2019-nCoV as SARS-CoV-2. For the purposes of this disclosure, the term “SARS-CoV-2” and “2019-nCoV” are considered to refer to the same virus and may be used interchangeably to refer to the etiologic agent for COVID-19. As used herein, these terms are also inclusive of separate variants of SARS-CoV-2, including variant B.1.1.7, variant 501Y.V2, and other variants that may emerge in the future.

[0131] As used herein, the term "kit" refers to any delivery system for delivering materials. In the context of reaction assays, such delivery systems include systems that allow for the storage, transport, or delivery of reaction reagents (e.g., oligonucleotides, enzymes, primer set(s), etc. in the appropriate containers) and/or supporting materials (e.g., buffers, written instructions for performing the assay etc.) from one location to another. For example, kits can include one or more enclosures (e.g., boxes) containing the relevant reaction reagents and/or supporting materials. As used herein, the term "fragmented kit" refers to a delivery system comprising two or more separate

containers that each contain a sub-portion of the total kit components. The containers may be delivered to the intended recipient together or separately. For example, a first container may contain an enzyme for use in an assay, while a second container contains oligonucleotides. Indeed, any delivery system comprising two or more separate containers that each contains a sub-portion of the total kit components are included in the term "fragmented kit." In contrast, a "combined kit" refers to a delivery system containing all of the components of a reaction assay in a single container (e.g., in a single box housing each of the desired components). The term "kit" includes both fragmented and combined kits.

[0132] Components described herein may be combined with one another and provided as such a combined kit or fragmented kit, or alternatively each component may be provided separately and utilized as desired by a user. For example, a treatment solution as described herein may be included in a kit or may be provided as a "stand-alone" item (in an appropriate container) for use as desired by the user.

[0133] As used herein, the term "patient" generally refers to any animal, for example a mammal, under the care, observation, or treatment of a healthcare provider, with particular reference to humans under the care of a primary care physician, infectious disease specialist, or other relevant medical professional that may diagnose or treat viral infections. For the purpose of the present application, a "patient" may be interchangeable with an "individual" or "subject." Accordingly, in some embodiments, the subject is a human patient. It should be appreciated, however, that a "subject" does not necessarily have to be a "patient," as that term is described herein. For example, a subject may be an asymptomatic carrier or uninfected person (or animal) being screened for one or more viruses. A "subject" can also be a non-human animal, such as a mink.

[0134] As used herein, the terms "real-time PCR" or "quantitative real-time PCR" or "qPCR" refer to the measurable amplification of nucleic acids via PCR in real time, typically by monitoring fluorescent probes in the reaction volume and enabling the optional quantitation of the PCR product. The terms "real-time" and "real-time continuous" are interchangeable and refer to a method where data collection occurs through periodic monitoring during the course of the amplification reaction. Thus, real-time methods combine amplification and detection into a single step. It should be appreciated that the data collection may occur through periodic monitoring during the course of PCR while the analysis of such data may occur later in time.

[0135] The terms "reverse transcription PCR" or simply "RT-PCR" are intended to include those PCR methods that first transcribe an RNA template (such as a viral RNA genomic template) into complementary DNA (cDNA) using an RNA-dependent DNA polymerase generally referred

to as a reverse transcriptase. The cDNA is then used by any of the DNA-dependent DNA polymerases commonly used in PCR methods as a template for PCR amplification of the target nucleic acid sequence. For ease of use within the specification, the terms "RT-PCR" and "RT-qPCR" may be used interchangeably, as it is understood by those having skill in the art that methods and reagents for monitoring amplicon production at the endpoint, such as is done in traditional PCR methods, can be adjusted such that amplicon production can be monitored during and/or between thermal cycles of PCR, such as is done in traditional qPCR methods.

[0136] Further, it should be appreciated that when the term "qPCR" is used herein, it does not necessarily exclude methods and/or kits that include an initial reverse transcription step. As such, any indication within the specification of a "qPCR" method, kit, array, and/or assay for performing qPCR is understood to include the same or similar method, kit, array, and/or assay having an initial reverse transcription step with any attendant reagents (*e.g.,* reverse transcriptase, buffers, dNTPs, salts, etc.).

[0137] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the present disclosure pertains.

[0138] Various aspects of the present disclosure, including devices, systems, and methods may be illustrated with reference to one or more embodiments or implementations, which are exemplary in nature. As used herein, the term "exemplary" means "serving as an example, instance, or illustration," and should not necessarily be construed as preferred or advantageous over other embodiments disclosed herein. In addition, reference to an "implementation" of the present disclosure or invention includes a specific reference to one or more embodiments thereof, and vice versa, and is intended to provide illustrative examples without limiting the scope of the invention, which is indicated by the appended claims rather than by the following description.

[0139] As used throughout this application the words "can" and "may" are used in a permissive sense (i.e., meaning having the potential to), rather than the mandatory sense (i.e., meaning must). Additionally, the terms "including," "having," "involving," "containing," "characterized by," as well as variants thereof (e.g., "includes," "has," "involves," "contains," etc.), and similar terms as used herein, including within the claims, shall be inclusive and/or open-ended, shall have the same meaning as the word "comprising" and variants thereof (e.g., "comprise" and "comprises"), and do not exclude additional un-recited elements or method steps, illustratively.

[0140] It will be noted that, as used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to a singular referent (e.g., "widget") includes one, two, or more

referents. Similarly, reference to a plurality of referents should be interpreted as comprising a single referent and/or a plurality of referents unless the content and/or context clearly dictate otherwise. For example, reference to referents in the plural form (e.g., "widgets") does not necessarily require a plurality of such referents. Instead, it will be appreciated that independent of the inferred number of referents, one or more referents are contemplated herein unless stated otherwise.

[0141] To facilitate understanding, like reference numerals (i.e., like numbering of components and/or elements) have been used, where possible, to designate like elements common to the figures. Specifically, in the exemplary embodiments illustrated in the figures, like structures, or structures with like functions, will be provided with similar reference designations, where possible. Specific language will be used herein to describe the exemplary embodiments. Nevertheless, it will be understood that no limitation of the scope of the disclosure is thereby intended. Rather, it is to be understood that the language used to describe the exemplary embodiments is illustrative only and is not to be construed as limiting the scope of the disclosure (unless such language is expressly described herein as essential).

[0142] Any headings used herein are for organizational purposes only and are not meant to be used to limit the scope of the description or the claims.

[0143] Various aspects of the present disclosure can be illustrated by describing components that are bound, coupled, attached, connected, and/or joined together. As used herein, the terms "bound," "coupled", "attached", "connected," and/or "joined" are used to indicate either a direct association between two components or, where appropriate, an indirect association with one another through intervening or intermediate components. In contrast, when a component is referred to as being "directly bound," "directly coupled", "directly attached", "directly connected," and/or "directly joined" to another component, no intervening elements are present or contemplated. Furthermore, binding, coupling, attaching, connecting, and/or joining can comprise mechanical and/or chemical association.

EXAMPLES

Example 1: Singleplex Assay for detecting SARS-CoV-2

[0144] An exemplary protocol for detecting SARS-CoV-2 from a biological sample via a singleplex assay was performed., as The assay used primers and FAM-labeled probes for detecting the ORF1ab, as well as additional primers and process for detecting S protein, and N protein coding sequences for SARS-CoV-2 selected from the primers and probes disclosed herein. The assay also used general-purpose components (e.g., master mix and other non-oligonucleotide components)

from TaqMan 2019-nCoV Assay Kit (Thermo Fisher Scientific, Catalog No. A47532). An optional VIC-labeled internal control directed to RNase P was also included. In a separate kit, the same primers/probes were included and used as positive controls to detect the target sequences from a synthetic DNA construct encoding the target sequences for ORF1ab, S protein, N protein, and RNase P.

[0145] The total nucleic acid content was isolated from samples collected via nasopharyngeal swab, nasopharyngeal aspirate, or bronchoalveolar lavage using the MagMAX Viral/Pathogen Nucleic Acid Isolation Kit (sold by Thermo Fisher Scientific under Cat. No. A42356) in accordance with the instructions provided therewith.

[0146] For each assay, the components in Table 4 were combined for the number of reactions, plus 10% overage:

Table 4. RT-qPCR Reaction Mix

Component	Volume / reaction
RT-qPCR Master Mix, CG (4X)	6.25 µL
2019 nCoV TaqMan Assay (20X) (FAM)	1.25 µL
TaqMan RNase P Assay, VIC dye/QSY assay (20X)	1.25 µL
Nuclease-free water	11.25 µL
Total Reaction Mix Volume	20.00 µL

[0147] The reaction mixes were vortexed for about 10–30 seconds and centrifuged briefly. For each reaction, the components in Table 5, below, were combined in a MicroAmp Optical 96-Well Reaction Plate (0.2 mL/well):

Table 5. RT-qPCR Reactions

Component	Volume / reaction
Reaction Mix (see Table 4)	20.00 µL
• Nucleic acid sample *or* • 1 µL 2019-nCoV Control construct + 4 µL PCR-grade water *or* • No template control (5 µL PCR-grade water)	5.00 µL
Total Reaction Volume	25.00 µL

[0148] The plate was sealed with a MicroAmp Optical Adhesive Film and vortexed briefly to mix the contents. The plate was centrifuged briefly to collect the contents at the bottom of the wells. The plate was loaded into a 7500 Real-Time PCR Instrument and the protocol in either Table 6 or Table 7 was run, depending on the respective RT-qPCR Master Mix used to create the reaction mix.

Table 6. RT-qPCR Protocol using TaqPath 1-Step RT-qPCR Master Mix

RT-qPCR Protocol *TaqPath 1-Step RT-qPCR Master Mix*				
Step	**Stage**	**# of cycles**	**Temp.**	**Time**
UNG incubation	1	1	25°C	2 min
Reverse transcription†	2	1	50°C†	15 min
Polymerase activation‡	3	1	95°C‡	2 min
Amplification	4	40	95°C	3 sec
			60°C	30 sec

† Preferably any temperature between 48°C – 55°C.

‡ RT inactivation, initial denaturation, and activation of DNAP.

Table 7. RT-qPCR Protocol using TaqMan Fast Virus 1-Step Master Mix

RT-qPCR Protocol *TaqMan Fast Virus 1-Step Master Mix*				
Step	**Stage**	**# of cycles**	**Temp.**	**Time**
Reverse transcription	1	1	50°C†	5 min
RT inactivation/initial Denaturation	2	1	95°C	20 sec
Amplification	3	40	95°C	3 sec
			60°C	30 sec

† Preferably any temperature between 48°C – 55°C.

[0149] The resulting data were analyzed using the included 7500 Software v2.3 because this program has updated algorithms with improved sensitivity for detecting low-copy samples. The analysis was performed using the Auto Baseline and Auto Threshold analysis settings of the aforementioned software. For each plate, the control reactions were confirmed to perform as expected (*i.e.,* the no template control had an undetermined C_t value and the positive control had a C_t value less than or equal to 30).

[0150] The C_t value for each individual assay was also analyzed in accordance with Table 8.

Table 8. Individual assay results guide

2019-nCoV assay (FAM)	**RNase P assay (VIC)**	**Interpreted Result**
$C_t < 37$	Any Value	Positive.
$37 \leq C_t < 40$	Any Value	Inconclusive. Repeat the test.
$C_t = 40$ or undetermined	$C_t < 40$	Negative.

[0151] The results for each tested sample was interpreted to have SARS-CoV-2 RNA present if either (i) any two of the three 2019-nCoV assays were positive or (ii) any one of the 2019-nCoV assays were positive in two different samples collected from the same subject. SARS-CoV-2 RNA was not present in the sample if all three of the 2019-nCoV assays were negative.

[0152] Exemplary results illustrating amplification plots and associated standard curves for ORF1ab are provided in FIGs. 2A and 2B, respectively. Similarly, an amplification plot and associated standard curve for S protein are provided in FIGs. 2C and 2D, respectively, and an amplification plot and associated standard curve for N protein are provided in FIGs. 2E and 2F, respectively.

Example 2: Multiplex Assay for detecting SARS-CoV-2

[0153] An exemplary protocol for detecting SARS-CoV-2 from a biological sample via a multiplex assay was performed. . The assay kit included primers and FAM-labeled probes for detecting ORF1ab (SEQ ID NOs: 160, 468 and 1049, respectively), primers and ABY-labeled probes for detecting S protein (SEQ ID NOs: 100, 337 and 864, respectively), and primers and VIC-labeled probes for detecting N protein (SEQ ID NOs: 211, 501and 833, respectively) coding sequences for SARS-CoV-2. A JUN-labeled internal positive control directed to either endogenous RNase P or an exogenous MS2 RNA template was also included. In a separate kit, the same primers/probes were included and used as positive controls to detect the target sequences from a synthetic DNA construct encoding the target sequences for ORF1ab, S protein, N protein, and RNase P/MS2 RNA. The remaining amplification reagents were obtained from the TaqPath™ COVID-19 Combo kit (Thermo Fisher Scientific, Catalog No. A47814).

[0154] The total nucleic acid content was isolated from samples collected via nasopharyngeal swab, nasopharyngeal aspirate, or bronchoalveolar lavage using the MagMAX Viral/Pathogen Nucleic Acid Isolation Kit (sold by Thermo Fisher Scientific under Cat. No. A42356) in accordance with the instructions provided therewith.

[0155] For each assay, the components in Table 9 were combined for the number of reactions, plus 10% overage:

Table 9. RT-qPCR Reaction Mix

Component	Volume / reaction
TaqPath 1-Step Multiplex Master Mix, CG (4X)	6.25 µL
nCoV Multiplex TaqMan Assay (20X)	1.25 µL
Internal positive control template	1.00 µL
Nuclease-free water	11.50 µL

Total Reaction Mix Volume	20.00 µL

[0156] The reaction mixes were vortexed for about 10–30 seconds and centrifuged briefly. For each reaction, the components in Table 10, below, were combined in a MicroAmp Optical 96-Well Reaction Plate (0.2 mL/well):

Table 10. RT-qPCR Reactions

Component	**Volume / reaction**
Reaction Mix (see Table 4)	20.00 µL
• Nucleic acid sample *or* • 1 µL 2019-nCoV Control construct + 4 µL PCR-grade water *or* • No template control (5 µL PCR-grade water)	5.00 µL
Total Reaction Volume	25.00 µL

[0157] The plate was sealed with a MicroAmp Optical Adhesive Film and vortexed briefly to mix the contents. The plate was centrifuged briefly to collect the contents at the bottom of the wells. reaction mixes were vortexed for about 10–30 seconds) and centrifuged briefly. The plate was loaded into a QuantStudio 5 Real-Time PCR System and the protocol in Table 11 was run.

Table 11. RT-qPCR Protocol for Multiplex Assay

Step	**Stage**	**# of cycles**	**Temp.**	**Time**
UNG incubation	1	1	25°C	2 min
Reverse transcription†	2	1	50°C†	15 min
Polymerase activation‡	3	1	95°C‡	2 min
Amplification	4	40	95°C	3 sec
			60°C	30 sec

† Preferably any temperature between 48°C – 55°C.

‡ RT inactivation, initial denaturation, and activation of DNAP.

[0158] The resulting data were analyzed using the QuantStudio Design and Analysis Software v1.5.1 included with the QuantStudio 5 Real-Time PCR System because this program has updated algorithms with improved sensitivity for detecting low-copy samples. For each plate, the control reactions were confirmed to perform as expected (*i.e.,* the no template control had an undetermined C_t value and the positive control had a C_t value less than or equal to 30).

[0159] The C_t value for each individual assay was also analyzed in accordance with Table 8.

Table 12. Individual assay multiplex assay results guide

	Ct < 37	37 ≤ Ct < 40	Ct = 40 or undetermined
ORF1ab (FAM)	Positive	Repeat test	Negative
N protein (VIC)	Positive	Repeat test	Negative
S protein (ABY)	Positive	Repeat test	Negative
Pos. Control (JUN)	Positive	Positive	Negative

[0160] The results for each tested sample was interpreted to have SARS-CoV-2 RNA present if either (i) any two of ORF1ab, S protein, or N protein were positive or (ii) any one of ORF1ab, S protein, or N protein were positive in two different samples collected from the same subject. SARS-CoV-2 RNA was not present in the sample if all three of ORF1ab, S protein, and N protein were negative.

Example 3: Robustness Testing of SARS-CoV-2 Detection Assays

[0161] To ensure the specificity observed when performing the singleplex and multiplex assays described in Examples 1 and 2, the robustness of each assay was tested based on RT-qPCR protocol, qPCR instrument, and RT-qPCR master mix used. Exemplary results shown in FIGs. 3A–3C illustrate robust assay performance under both the 7500 Standard Protocol and the 7500 Fast Protocol and indicate that either protocol can be used effectively to identify samples with SARS-CoV-2 RNA.

[0162] The robustness of assay performance between qPCR instruments was also confirmed. As shown in FIGs. 4A–4C, the disclosed SARS-CoV-2 assays are likely to be effective in identifying SARS-CoV-2 RNA within a sample regardless of whether the 7500 Instrument or the QuantStudio 5 system is used.

[0163] Although assay robustness was not predicted to vary with respect to the type of master mix used, the results shown in FIGs. 4A–4C were specific to assays using TaqMan Fast Virus 1-Step Master Mix. Thus, assay robustness with respect to master mix was also tested. As shown in FIGs. 5A–5C, both the TaqPath 1-Step Master Mix and the Fast Virus 1-Step Master Mix show robust assay performance run on the Quant Studio 5 instrument, making either master mix effective for identifying samples containing SARS-CoV-2 RNA.

[0164] A summary of these experiments is shown in Table 13, below, indicating that the disclosed assays are robust enough to identify samples having SARS-CoV-2 RNA regardless of the qPCR master mix, qPCR protocol, or qPCR instrument used.

Table 13. Summary of assay robustness survey

Target	Applied Biosystems Instrument	Applied Biosystems Master Mix	Slope	R^2	Efficiency

ORF1ab	7500 Real-Time PCR System	TaqMan Fast Virus 1-Step Master Mix (Standard)	-3.47	0.998	94.15%
	QuantStudio™ 5 Real-Time PCR System	TaqMan Fast Virus 1-Step Master Mix (Standard)	-3.321	0.998	100.02%
		TaqPath 1-Step RT-qPCR Master Mix, CG	-3.385	0.999	97.42%
N protein	7500 Real-Time PCR System	TaqMan Fast Virus 1-Step Master Mix (Standard)	-3.443	0.999	95.18%
	QuantStudio™ 5 Real-Time PCR System	TaqMan Fast Virus 1-Step Master Mix (Standard)	-3.417	0.999	96.18%
		TaqPath 1-Step RT-qPCR Master Mix, CG	-3.324	0.995	99.91%
S Protein	7500 Real-Time PCR System	TaqMan Fast Virus 1-Step Master Mix (Standard)	-3.364	0.999	98.26%
	QuantStudio™ 5 Real-Time PCR System	TaqMan Fast Virus 1-Step Master Mix (Standard)	-3.408	0.997	96.51%
		TaqPath 1-Step RT-qPCR Master Mix, CG	-3.319	0.998	100.11%

Example 4: Specificity test of singleplex and multiplex assays for SARS-CoV-2

[0165] Nucleic acids of 22 viruses and bacteria (listed below) were extracted with MagMAX Viral/Pathogen Ultra Nucleic Acid Isolation Kit (sold by Thermo Fisher Scientific under Catalog No. A42356) on KingFisher and used for this test. Extracted RNA and DNA were pre-amplified with TaqPath 1-Step RT-qPCR Master Mix, CG and a PreAmp pool containing primers for both SARS-CoV-2 and MEGAPLEX PREAMP PRIMERS, RTM (sold by Thermo Fisher Scientific Catalog No. A41374). Pre-amplification was carried out using the thermal cycling protocol of described in Table 14 below.

Table 14. Pre-amplification protocol

Step	**Stage**	**# of cycles**	**Temp.**	**Time**
UNG incubation	1	1	25°C	2 min
Reverse transcription†	2	1	50°C†	30 min
Polymerase activation‡	3	1	95°C‡	2 min
Amplification	4	14	95°C	15 sec
			60°C	2 min
Denaturation	5	1	99°C	10 min

† Preferably any temperature between 48°C – 55°C.

‡ RT inactivation, initial denaturation, and activation of DNAP.

[0166] The product of the PreAMP reaction was diluted in a 1:10 ratio with water (preferably RT-PCR grade water), and 5 µL of the diluted PreAmp reaction was added to a 25 µL reaction volume for singleplex and multiplex reactions containing RT-PCR enzymes and assay reagents.

The recipe for singleplex reactions is provide in Table 15, and the recipe for multiplex reactions is provided in Table 16.

[0167] 5 µL of diluted PreAmp reaction (*i.e.,* sample), and 11 µL of water (preferably RT-PCR grade water). Reactions were run on QS5 instrument following recommended protocols of respective master mixes. Three replicates were performed for each sample.

[0168]

Table 15. RT-qPCR Mix for singleplex reactions

Component	Volume / reaction
TaqPath 1-Step Multiplex Master Mix, CG (4X)	6.25 µL
SARS-CoV-2 viral gene singleplex assay (20X)	1.25 µL
RNase P positive control assay (20X)	1.25 µL
Nuclease-free water	11.25 µL
Total Reaction Mix Volume	20.00 µL

Table 16. RT-qPCR Mix for multiplex reactions

Component	Volume / reaction
TaqPath 1-Step Multiplex Master Mix, (No ROX)	6.25 µL
SARS-CoV-2 viral gene multiplex assay (20X)	1.25 µL
Internal positive control template	1.00 µL
Nuclease-free water	11.50 µL
Total Reaction Mix Volume	20.00 µL

[0169] The reaction mixes were each vortexed for about 10–30 seconds and centrifuged briefly. The preamplified and diluted samples for each of 22 viruses and bacteria were used for specificity testing. All organisms were obtained from ZeptoMetrix with exception of Coronavirus strain HKU1, which was a clinical isolate. For each reaction mixture and genomic sample to be tested, the components in Table 17, below, were combined within a MicroAmp Optical 96-Well Reaction Plate (0.2 mL/well) in triplicate:

Table 17. RT-qPCR Reactions

Component	Volume / reaction
Reaction Mix (see Tables 15 and 16)	20.00 µL
• Nucleic acid sample *or* • 1 µL 2019-nCoV Control construct + 4 µL PCR-grade water *or* • No template control (5 µL PCR-grade water)	5.00 µL
Total Reaction Volume	25.00 µL

[0170] Results: None of the 22 organisms tested show cross reactivity to either singleplex or multiplex assays for SARS-CoV-2 detection, as summarized in Table 18 below. Thus, the assays are specific.

Table 18. Specificity test results for 2019-nCoV singleplex and multiplex assays

	Amplification Detected (singleplex assay | multiplex assay)			
Sample Tested	**ORF1ab**	**N Protein**	**S Protein**	**Multiplex control**
Coronavirus 229E	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3
Coronavirus HKU	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3
Coronavirus NL63	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3
Coronavirus OC43	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3
Flu A (H3)	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3
Flu A (H1-2009)	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3
Flu B (Pan)	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3
RSV A	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3
RSV B	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3
Rhinovirus	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3
Human parainfluenza virus 1	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3
Human parainfluenza virus 2	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3
Human parainfluenza virus 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3
Human parainfluenza virus 4	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3
Enterovirus (Pan)	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3
Enterovirus (D68)	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3
Human metapneumovirus	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3
Adenovirus	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3
Mycoplasma pneumoniae	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3
Legionella pneomophila	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3
Bordetella pertussis	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3
Bordetella parapertussis	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3
No template control	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3
2019nCoV positive control	0 of 3 | 0 of 3	0 of 3 | 0 of 3	0 of 3 | 0 of 3	N/A | 3 of 3

Example 5: Mixing RT-PCR reaction plates

[0171] To ensure proper analysis of SARS-CoV-2 research samples, it is essential to mix the RT-qPCR reaction properly by vortexing the plate. Failure to do so can result in Optical Mixing, a phenomenon that is likely to occur when the sample volume exceeds 20% of the PCR reaction volume. Optical Mixing can lead to RTPCR baseline instability, resulting in QC failure of entire plates and potential false classification of samples.

[0172] Mixing Protocol:

[0173] After master mix, assay, water, samples, and controls were added to the RT-PCR reaction plate, the plate wells were sealed with a MicroAmp Optical Adhesive Film. The

MicroAmp Adhesive Film Applicator was used to make sure all wells were sealed completely. The MicroAmp Optical Adhesive Cover uses a pressure- sensitive adhesive backing to adhere the cover to the optical 96- or 384-well plate. Using enough force to activate the pressure-sensitive adhesive will prevent evaporation from the wells.

[0174] The speed of a vortex mixer, such as the Vortex-Genie 2 from Scientific Industries, was set to the highest setting with the activation mode set to "Touch." The vortex mixer was also outfitted with a platform rather than a tube cup.

[0175] The plate was contacted with the vortex mixer and held in contact therewith for 10–15 seconds while allowing the vortex platform to move vigorously and cause the reaction mix to move freely in the wells. Too much or too little pressure applied to the vortex platform reduced mixing efficiency. The plate was moved around during vortexing to ensure that contact with the platform has been made at all four quadrants and the center of the plate for equal time.

[0176] Experimental Design:

[0177] In a first set of experiments, two identical 96-well plates were created. A first plate was vortexed for 30 seconds at maximum speed on a Vortex-Genie 2, and the other plate was not mixed at all. Each plate contained triplicate reactions of extracted contrived positive samples and negative samples. Contrived positive samples consisted of pooled nasopharyngeal specimens spiked with SARS-CoV-2 viral RNA at 9X, 3X, or 1X the Limit of Detection (2,250 GCE/mL, 750 GCE/mL and 250 GCE/mL, respectively). Samples were extracted with either the MagMAX Viral/Pathogen Nucleic Acid Isolation Kit and a 400-µL specimen volume or the MagMAX Viral/Pathogen II Nucleic Acid Isolation Kit and a 200-µL specimen volume, and both extraction workflows were run on the same RT-PCR plate.

[0178] In a second set of experiments, two identical 384-well plates were created; one plate was vortexed for 10 seconds at maximum speed on a Vortex-Genie 2, and the other plate was not mixed at all. Each plate contained 48 replicate reactions of extracted SARS-CoV-2 viral RNA at 10 GCE/reaction plus the MS2 Internal Control. RT-PCR runs were performed on an Applied Biosystems QuantStudio 7 Flex system with a 384-well block.

[0179] Results:

[0180] The 96-well and 384-well plates that were not mixed demonstrated steep downward slopes in the fluorescent signal during the early cycles of the thermal protocol. By contrast, the 96-well and 384-well plates that were mixed revealed that proper mixing produces flatter baselines for the same conditions. Thus, compared with no mixing, vortexing for 10 – 30 seconds produces flatter baselines irrespective of extraction protocol, plate type, or sample type. Because falling

baselines can produce plate failures or inaccurate results, vortex mixing appears important for achieving reliable, specific results.

Example 6: SARS-CoV-2, Flu A/B, RSV Multiplex Assay

[0181] An exemplary protocol for detecting the presence of genomic nucleic acid associated with SARS-CoV-2, Flu A/B, or RSV from a biological sample via a multiplex assay was performed using primers and FAM-labeled probe for detecting target sequences from Flu (Influenza) Types A and B genomes (SEQ ID NOs: 252, 253, 257, 505, 506, 1296 and 1297); primers and VIC-labeled probe for detecting SARS-CoV-2 target sequences from the S gene in SARS-CoV-2 (SEQ ID NOs: 100, 337 and 864), and N protein (SEQ ID NOs: 211, 510 and 833), primers and ABY-labeled probes for detecting target sequences specific for the RSV viral genome (SEQ ID Nos: 254, 255, 507, 508, 1298 and 1299), and primers and JUN-labeled probes for an internal positive control directed to an exogenous MS2 RNA template (SEQ ID NOS: 206, 509 and 1300). The primer of SEQ ID NO: 510 used for this experiment is similar to the primer of SEQ ID NO: 501, but also includes an additional "A" nucleotide residue at the 3' end. The addition of this residue was found to be helpful in reducing artifacts formed during PCR reactions containing certain additional primers and probes.

[0182] In separate wells, the same primers/probes were included and used together with a synthetic positive control construct encoding the target sequences for identifying SARS-CoV-2, Flu (A and B), and RSV.

[0183] The total nucleic acid content was isolated from samples collected via nasopharyngeal swab, nasopharyngeal aspirate, or bronchoalveolar lavage using the MagMAX Viral/Pathogen Nucleic Acid Isolation Kit (sold by Thermo Fisher Scientific under Cat. No. A42356) in accordance with the instructions provided therewith.

[0184] The components in one of Tables 19A–19C were combined to make the RT-PCR Reaction Mix for the total number of reactions, plus 10% overage:

Table 19A. RT-qPCR Reaction Mix

Component	Volume / reaction
TaqPath 1-Step Multiplex Master Mix, CG (4X)	6.25 µL
Primers and probes	1.25 µL
Total Reaction Mix Volume	7.50 µL

Table 19B. RT-qPCR Reaction Mix

Component	Volume / reaction
TaqPath 1-Step Multiplex Master Mix, CG (4X)	6.25 µL

Primers and probes	1.25 µL
Nuclease-free Water	7.5 µL
Total Reaction Mix Volume	15.0 µL

Table 19C. RT-qPCR Reaction Mix (400-µL sample input volume)

Component	Volume / reaction
TaqPath 1-Step Multiplex Master Mix, CG (4X)	6.25 µL
Primers and probes	1.25 µL
Nuclease-free Water	12.50 µL
Total Reaction Mix Volume	20.0 µL

[0185] The reaction mixes were vortexed for about 10–30 seconds) and centrifuged briefly. For each reaction, the components in Table 20A–20D, below, were combined in a MicroAmp Optical 96-Well Reaction Plate:

Table 20A. RT-qPCR Reactions (0.2 mL/well)

	Volume / reaction		
Component	**RNA Sample**	**Pos. Control**	**Neg. Control**
Reaction Mix (see Table 19A)	7.5 µL	7.5 µL	7.5 µL
Purified sample RNA	17.5 µL	—	—
Diluted TaqMan SARS-CoV-2, Flu A/B, RSV RNA Control	—	17.5 µL	—
Negative Control (from RNA extraction)	—	—	17.5 µL
Total Reaction Volume	25.0 µL	25.0 µL	25.0 µL

Table 20B. RT-qPCR Reactions (0.2 mL/well)

	Volume / reaction		
Component	**RNA Sample**	**Pos. Control**	**Neg. Control**
Reaction Mix (see Table 19A)	7.5 µL	7.5 µL	7.5 µL
Purified sample RNA	17.5 µL	—	—
Diluted TaqMan SARS-CoV-2, Flu A/B, RSV RNA Control	—	2.0 µL	—
Nuclease-free Water	—	15.5 µL	—
Negative Control (from RNA extraction)	—	—	17.5 µL
Total Reaction Volume	25.0 µL	25.0 µL	25.0 µL

Table 20C. RT-qPCR Reactions (0.2 mL/well)

	Volume / reaction		
Component	**RNA Sample**	**Pos. Control**	**Neg. Control**

Reaction Mix (see Table 19B)	15.0 µL	15.0 µL	15.0 µL
Purified sample RNA	10.0 µL	—	—
Diluted TaqMan SARS-CoV-2, Flu A/B, RSV RNA Control	—	2.0 µL	—
Nuclease-free Water	—	8 µL	—
Negative Control (from RNA extraction)	—	—	10.0 µL
Total Reaction Volume	25.0 µL	25.0 µL	25.0 µL

Table 20D. RT-qPCR Reactions (0.4 mL/well)

	Volume / reaction		
Component	**RNA Sample**	**Pos. Control**	**Neg. Control**
Reaction Mix (see Table 19C)	20.0 µL	20.0 µL	20.0 µL
Purified sample RNA	5 µL	—	—
Diluted TaqMan SARS-CoV-2, Flu A/B, RSV RNA Control	—	2.0 µL	—
Nuclease-free Water	—	3.0 µL	—
Negative Control (from RNA extraction)	—	—	5.0 µL
Total Reaction Volume	25.0 µL	25.0 µL	25.0 µL

[0186] The plate was sealed with a MicroAmp Optical Adhesive Film and vortexed briefly to mix the contents. The plate was centrifuged briefly to collect the contents at the bottom of the wells. The plate was loaded into a 7500 Real-Time PCR Instrument and the protocol in Table 21 was run.

Table 21. RT-qPCR Protocol for Multi-Pathogen Multiplex Assay

Step	**Stage**	**# of cycles**	**Temp.**	**Time**
UNG incubation	1	1	25°C	2 min
Reverse transcription†	2	1	53°C†	10 min
Preincubation	3	1	85°C	10 min
Polymerase activation	4	1	95°C‡	2 min
Amplification	5	46	95°C	3 sec
			60°C	30 sec

† Preferably any temperature between 48°C – 55°C.

[0187] The results for each tested sample having amplified product in the positive control and no amplified product in the negative control were interpreted to have: (i) SARS-CoV-2 RNA present if the VIC signal was positive, (ii) Flu A/B if the FAM signal was positive, and/or (iii) RSV if the ABY signal was positive.

Example 7

[0188] A 4-plex RT-qPCR assay was developed in 1-tube, for nucleic acid detection of SARS-CoV-2, Flu A, and Flu B viruses along with a process control. This SARS-CoV-2 portion of the 4-plex assay targets both S protein and N protein regions having higher specificity and exhibiting lower risk for mutation. The primers targeting the S protein region were SEQ ID NOs: 100 and 337, with the associated probe of SEQ ID NO: 864. The primers targeting the N protein region were SEQ ID NOs: 211 and 510, with the associated probe of SEQ ID NO: 833. A proprietary bioinformatics pipeline was used to design specific assays for Flu A and Flu B with great coverage. The resulting strain coverage for the primer and probe sets was 99.9% for SARS-CoV-2 with 35,833 high quality complete sequences. The resulting strain coverage for the primer and probe sets for Flu A and Flu B is 6,730/6,854 or 98.2%, and 3,105/3,127 or 99.3%, respectively. Assays were tested with qPCR instruments such as QS5 and 7500 Fast Dx using a modified RT-PCR protocol based on TaqPath COVID-19 Combo Kit (Appendix 2) and using the same master mix, water and dNTPs provided therein.

[0189] DNA, *in vitro* transcribed RNA, genomic RNA, and viral organism controls are used at different stages of feasibility testing and development. DNA and *in vitro* transcribed RNA controls include SARS-CoV-2, Flu A and Flu B. Viral RNA and viral organism controls include SARS-CoV-2, genomic RNA, and gamma irradiation inactivated virus Influenza A: H1N1 (Brisbane/59/2007) and H3N2 (Perth/16/2009), and Influenza B: Victoria lineage (Wisconsin/01/2010) and Yamagata lineage (Florida/04/2006).

[0190] PCR thermal cycling protocol was done according to our previous TaqPath COVID-19 Combo Kit with two modifications: (1) a preincubation step (85°C, 10min) was added after RT (preincubation reduces ABY channel's abnormal amplification curves); and (2) the number of cycles was increased from 40 to 46 (increases ΔRn of true amplification above maximum crosstalk level).

[0191] Criteria for the analytical validation studies:

[0192] The workflow limit of detection (LoD) will be established as the lowest concentration of GCE of the virus that can be detected ≥ 95% of the time (*e.g.*, ≥ 19/20 replicates).

[0193] Reactivity/inclusivity: 100% of strains tested must by detected by the assay; Strains not detected at 3X LoD will be retested at higher concentrations until 100% hit rate is achieved; 100% of replicates must be positive.

[0194] Interference substance: A substance will be determined to be non-interfering at the tested concentration if 100% of replicates produce the expected results for a given interferent.

[0195] Competitive interference: At least 95% of positive samples at 4X LoD or lower produce a result of Positive in the presence of competitors at 1,000X LoD or higher ΔC_t = Mean Combination C_t – Mean Single Target $C_t \leq 1.5$ Cycles

[0196] The 4-plex real-time PCR assays can simultaneously detect and differentiate SARS-CoV-2, Flu A, and Flu B viral nucleic acids. In the tested examples, Flu A primers and probe sets were associated with the FAM channel, SARS-CoV-2 N protein and S protein primers and probe sets were associated with the VIC channel, Flu B primers and probe sets were associated with the ABY channel, and the internal control MS2 primers and probe sets were associated with the JUN channel.

[0197] One strain of SARS-CoV-2, two strains of Flu A (one H1N1 and one H3N2) and two strains of Flu B (one Victoria lineage and one Yamagata lineage) were examined for workflow LoD. Viral RNA extraction using MagMAX Viral/Pathogen II (MVP II) Nucleic Acid Isolation Kit and KingFisher Flex 96 Deep Well. RT-qPCR was performed on the 7500 Fast Dx Real-Time PCR system and the QuantStudio 5 Real-Time PCR Instrument. The results from the workflow limits of detection are provided in Table 22 below.

Table 22. Workflow Limits of Detection (LoD)

Virus	Subtype	Strain	Limit of Detection	
SARS-CoV-2	N/A	USA-WA1/2020	100 GCE/mL	0.16 $TCID_{50}$/mL
Flu A (Perth)	H3N2	A/Perth/16/2009	200 GCE/mL	0.00249 $TCID_{50}$/mL
Flu A (Brisbane)	H1N1	A/Brisbane/59/2007	500 GCE/mL	0.00159 $TCID_{50}$/mL
Flu B (Florida)	Yamagata	B/Florida/04/2006	500 GCE/mL	0.0588 $TCID_{50}$/mL
Flu B (Wisconsin)	Victoria	B/Wisconsin/01/2010	1000 GCE/mL	0.00560 $TCID_{50}$/mL

[0198] The primers and probes used in the 4-plex assay did not cross-react with any of the 41 respiratory pathogens tested and listed in Table 23 below.

Table 23. RT-qPCR Protocol for Multi-Pathogen Multiplex Assay

Bacteria and Fungi	Viruses	Purified DNA
Chlamydia pneumoniae	Adenovirus	*Bacillus anthracis*
Bordetella pertussis	Enterovirus	*Leptospira interrogans*
Candida albicans	Coronavirus 229E	*Moraxella catarrhalis*
Corynebacterium diphtheriae	Coronavirus NL63	*Mycobacterium tuberculosis*
Haemophilus influenzae	Coronavirus OC43	*Streptococcus pneumoniae*
Legionella non- pneumophila	Human Metapneumovirus	*Streptococcus pyogenes*
Legionella pneumophila	Influenza C virus	Coronavirus HKU1
Neisseria elongata	MERS-CoV	*Chlamydia psittaci*
Neisseria meningitidis	Parainfluenza virus 1	*Coxiella burnetii*
Pseudomonas aeruginosa	Parainfluenza virus 2	

Staphylococcus aureus	Parainfluenza virus 3	
Staphylococcus epidermidis	Parainfluenza virus 4	
Streptococcus salivarius	Parechovirus	
Pneumocystis carinni	RSV A	
Mycoplasma pneumoniae	RSV B	
	Rhinovirus	
	SARS-CoV	

[0199] Possible interference substances were tested to demonstrate the ability of this 4-plex test to detect each target virus at a low concentration in the presence of potentially interfering substances and to demonstrate that the potential interferents alone do not produce false positive results. Blood, corticosteroid nasal spray, nasal gel, homeopathic allergy relief nasal spray, throat lozenges, Oseltamivir, antibiotic ointment, and systemic antibiotics tested show no interference. Afrin Original nasal spray showed interference at 10% v/v, so it was titrated down to find the concentration at which no inhibition was observed. The maximum, non-inhibitory concentration was 0.6% on both instruments and all viruses tested. The results are summarized in Table 24 below.

Table 24. RT-qPCR Protocol for Multi-Pathogen Multiplex Assay

Potential Interfering Substance	**Final Concentration**	**Result**
Mucin	0.1 mg/mL	Pass
Blood	1% v/v	Pass
Nasal spray	10% v/v	Pass
Nasal corticosteroid	5 µg/mL	Pass
Nasal gel	1% w/v	Pass
Homeopathic allergy relief medicine	10% v/v	Pass
Throat lozenges	1% w/v	Pass
Oseltamivir phosphate	33 µg/mL	Pass
Antibiotic, nasal ointment	5 µg/mL	Pass
Systemic Antibiotic	0.6 mg/mL	Pass

[0200] A competitive interference study was also performed to assess the ability of the 4-plex SARS-CoV-2, Flu A, and Flu B test to detect each target virus at a low concentration in the presence of another target virus at a high concentration. Competitive interference testing was performed using contrived NP samples with SARS-CoV-2, Flu A, and Flu B with one virus at a concentration less than or equal to three times its LoD and the other tested virus at a concentration greater than or equal to 10^5 $TCID_{50}$/mL. A summary of the results is in Table 25 below.

Table 25. Competitive Interference Results

Virus combination	**Concentration Passed**
SARS-CoV-2^{lo} $FluB^{hi}$	3X LoD

$FluB^{lo}$ $SARS\text{-}CoV\text{-}2^{hi}$	3X LoD
$FluA^{lo}$ $FluB^{hi}$	3X LoD
$FluB^{lo}$ $FluA^{hi}$	3X LoD
$SARS\text{-}CoV\text{-}2^{lo}$ $FluA^{lo}$ $FluB^{lo}$	3X LoD
$SARS\text{-}CoV\text{-}2^{lo}$ $FluA^{hi}$	4X LoD
$FluA^{lo}$ $SARS\text{-}CoV\text{-}2^{hi}$	4X LoD

Example 8: Limit of Detection

[0201] The purpose of this study was to establish the Limits of Detection (LoD) for SARS-CoV-2, influenza A, influenza B, RSV A, and RSV B for use on a 96-well real-time PCR platform. The experiment uses primers targeting the S protein and N protein of SARS-CoV-2. The primers targeting the S protein region were SEQ ID NOs: 100 and 337, with the associated probe of SEQ ID NO: 864. The primers targeting the N protein region were SEQ ID NOs: 211 and 510, with the associated probe of SEQ ID NO: 833. The LoD will be established as the lowest number (concentration) of Genomic Copy Equivalents (GCE) of each virus that can be detected at least 95% of the time.

[0202] Individual LoD for each of the viruses detected by the COVID/Flu/RSV test were determined using contrived specimens comprising inactivated SARS-CoV-2 virus and live influenza A and B, and respiratory syncytial viruses spiked at various levels into pooled nasopharyngeal (NP) swab specimens. Inactivated SARS-CoV-2 virus was obtained from BEI Resources (PN NR-52287, LN 70033322). Two strains of live influenza A virus (referred to as Perth and Brisbane, respectively) were obtained from ZeptoMetrix: Influenza A H3N2 (strain A/Perth/16/2009; PN 0810251CF, LN 313219) and Influenza A H1N1 (strain A/Brisbane/59/2007; PN 0810244CF, LN 323919). Two strains of live influenza B virus (referred to as Florida and Wisconsin, respectively) were obtained from ZeptoMetrix: Influenza B Yamagata lineage (strain B/Florida/04/2006; PN 0810255CF, LN 312479) and Influenza B Victoria lineage (strain B/Wisconsin/01/2010; PN 0810241CF, LN 324993). One strain of RSV A virus was obtained from ZeptoMetrix (PN 0810040ACF, LN 324695). One strain of RSV B virus was obtained from ZeptoMetrix (PN 0810480CF, LN 322742). The quantitated GCE/mL values for the stock materials were determined by dPCR, and dilutions were appropriately formulated based on this information.

[0203] Sample extraction was performed using the MagMAX Viral/Pathogen II Nucleic Acid Isolation Kit and KingFisher Flex system. Samples were tested using the COVID/Flu/RSV test with the TaqPath 1-Step Multiplex Master Mix (No ROX) on the Applied Biosystem 7500 Fast Real-Time PCR Instrument. The study was conducted in three phases: a preliminary LoD was

determined in Phase I of the study, a refined LoD was determined in Phase II, and the LoD was confirmed in Phase III. A preliminary LoD was determined in Phase I by testing contrived samples with known GCEs at six levels, beginning from sample extraction. Preliminary LoD testing was performed with three replicate contrived samples at each GCE level. Following determination of the preliminary LoD, Phase II refined the LoD with five replicate specimens per five levels at, below, and above, the preliminary LoD, and Phase III confirmed the LoD with at least 95% detection across 20 replicate samples.

[0204] Testing was performed under manufacturer-recommended conditions for the KingFisher Flex and Real-Time PCR instruments. Room temperature steps were performed in laboratories with a temperature between 15°C and 30°C.

[0205] All remaining non-oligonucleotide reagents (e.g., master mix, water, dNTPs, etc.) were obtain from the TaqPath™ COVID-19 Combo kit (Thermo Fisher Scientific, Catalog No. A47814) and RT-PCR was conducted according to the protocol supplied therewith.

[0206] Procedure

[0207] A pool of at least 175 mL of NP specimens was prepared and divided equally for the five viruses; the same pool of each virus was used for the entire study phase.

[0208] Inactivated SARS-CoV-2 virus was obtained and diluted in Nucleic Acid Dilution Solution (NADS) or VTM just prior to extraction in accordance with Table 26; each dilution was mixed gently but thoroughly. To prevent cross-contamination, gloves were changed after making the intermediate dilutions and before beginning the LoD dilutions. Calculations in the following table were based on a stock concentration of 1.75×10^9 GCE/mL (2.8×10^9 $TCID_{50}$/mL); if inactivated SARS-CoV-2 virus was not formulated at this concentration, the calculations were amended to produce the following concentrations.

Table 26.

Dilution ID	Final Conc. Virus	Vol. Virus Stock	Vol. Diluent	Vol. NP Pool
Intermed-1	5.0×10^7 GCE/mL	2.0 µL of stock	68.0 µL	--
Intermed-2	5.0×10^5 GCE/mL	10 µL of Intermed-1	990 µL	--
Intermed-3	5.0×10^3 GCE/mL	20 µL of Intermed-2	1980 µL	--
1	2.5×10^3 GCE/mL	1500 µL of Intermed- 3	--	1500 µL
2	1.25×10^3 GCE/mL	1500 µL of Dilution 1	--	1500 µL
3	5.0×10^2 GCE/mL	1200 µL of Dilution 2	--	1800 µL
4	2.5×10^2 GCE/mL	1500 µL of Dilution 3	--	1500 µL
5	100 GCE/mL	1200 µL of Dilution 4	--	1800 µL
6	50 GCE/mL	1500 µL of Dilution 5	--	1500 µL
7	0 GCE/mL	--	--	1500 µL

[0209] Live RSV A, RSV B, Flu A and Flu B viruses were obtained and viral genomic RNA was quantitated by digital PCR. Live RSV A, RSV B, Flu A virus, and Flu B virus were diluted to the same concentrations based upon the concentrations determined by digital PCR. Either NADS or VTM were used as the virus diluent.

[0210] LoD Phase I

[0211] Each test level included at least three replicate extractions. The preliminary LoD was the lowest concentration at which all three extraction replicates produce a result of Positive.

[0212] Using the specimens prepared in steps 12.2 and 12.3, Contrived samples were extracted in triplicate from the specimens prepared and were tested by RT-qPCR on a 7500 Fast platform. The preliminary LoD was calculated as the lowest concentration (highest Test Level ID) at which all three extraction replicates produced a result of Positive.

[0213] On the 7500 Fast real-time PCR instrument, the Preliminary LoD for SARS-CoV-2 was established in Phase I as 50 GCE/mL, the Preliminary LoD for Flu A (Perth) was established in Phase I as 250 GCE/mL, the Preliminary LoD for Flu A (Brisbane) was established in Phase I as 384 GCE/mL, the Preliminary LoD for Flu B (Florida) was established in Phase I as 500 GCE/mL, the Preliminary LoD for Flu B (Wisconsin) was established in Phase I as 250 GCE/mL, the Preliminary LoD for RSV A was established in Phase I as 50 GCE/mL, and the Preliminary LoD for RSV B was established in Phase I as 250 GCE/mL.

[0214] LoD Phase II

[0215] Each test level included at least five replicate extractions. The refined LoD was the lowest concentration at which all five extraction replicates produced a result of Positive.

[0216] Contrived samples were prepared to the levels in Table 27 below with at least five replicates per Test Level. Virus dilutions compatible with the concentrations being tested were formulated, and contrived samples were extracted and tested by RT-qPCR on a 7500 Fast platform. The refined LoD was calculated for each virus as the lowest concentration (highest Test Level ID) at which all five extraction replicates produced a result of Positive.

Table 27.

Test Level ID	Final Concentration Virus	Replicates
1	3X Preliminary LoD	5
2	2X Preliminary LoD	5
3	Preliminary LoD	5
4	0.5X Preliminary LoD	5
5	0.33X Preliminary LoD	5
6	0 GCE	5

[0217] On the 7500 Fast real-time PCR instrument, the Refined LoD for SARS-CoV-2 was established in Phase II as 50 GCE/mL, the Refined LoD for Flu A (Perth) was established in Phase II as 250 GCE/mL, the Refined LoD for Flu A (Brisbane) was established in Phase II as 768 GCE/mL, the Refined LoD for Flu B (Florida) was established in Phase II as 1000 GCE/mL, the Refined LoD for Flu B (Wisconsin) was established in Phase II as 250 GCE/mL, the Refined LoD for RSV A was established in Phase II as 150 GCE/mL, and the Refined LoD for RSV B was established in Phase II as 200 GCE/mL.

[0218] LoD Phase III

[0219] The refined LoD determined in Phase II was confirmed with at least 20 replicate extractions. The LoD was confirmed if at least 19 of 20 extraction replicates produced a result of Positive.

[0220] Contrived samples were prepared to the levels in Table 28 below with at least twenty replicates per Test Level. Virus dilutions compatible with the concentrations being tested were formulated, and contrived samples were extracted and tested by RT-qPCR on a 7500 Fast platform. The LoD for each specimen was confirmed if at least 95% of extraction replicates produced a result of Positive. If the LoD was not confirmed for any of the five viruses, Phase III was repeated for that virus at a higher concentration.

Table 28.

Test Level ID	Final Concentration Virus	Replicates
1	Refined LoD	20
2	0 GCE	1

[0221] On the 7500 Fast real-time PCR instrument, the Confirmed LoD for SARS-CoV-2 established in Phase III was 50 GCE/mL, the Confirmed LoD for Flu A (Perth) established in Phase III was 350 GCE/mL, the Confirmed LoD for Flu A (Brisbane) established in Phase III was 384 GCE/mL, the Confirmed LoD for Flu B (Florida) established in Phase III was 1250 GCE/mL, the Confirmed LoD for Flu B (Wisconsin) established in Phase III was 350 GCE/mL, the Confirmed LoD for RSV A established in Phase III was 200 GCE/mL, and the Confirmed LoD for RSV B established in Phase III was 200 GCE/mL.

[0222] LoDs for the TaqPath COVID-19, Flu A / Flu B, RSV Combo Kit (COVID/Flu/RSV test) were established using the 7500 Fast real-time PCR instrument (*e.g.,* 7500 Fast Dx for 96-well plates using 17.5 µL of reaction volume input and QS7 Flex for 384-well plates using 14 µL of reaction volume input); the results are summarized in Table 29 below.

Table 29. Summary Workflow Limits of Detection (LoD)

Virus	Subtype	Strain	Limit of Detection	
SARS-CoV-2	N/A	USA-WA1/2020	50 GCE/mL	0.00824 $TCID_{50}$/mL
Flu A (Perth)	H3N2	A/Perth/16/2009	350 GCE/mL	0.0221 $TCID_{50}$/mL
Flu A (Brisbane)	H1N1	A/Brisbane/59/2007	384 GCE/mL	0.00123 $TCID_{50}$/mL
Flu B (Florida)	Yamagata	B/Florida/04/2006	1250 GCE/mL	0.147 $TCID_{50}$/mL
Flu B (Wisconsin)	Victoria	B/Wisconsin/01/2010	350 GCE/mL	0.00424 $TCID_{50}$/mL
RSV A	N/A	A/2006	200 GCE/mL	0.0136 $TCID_{50}$/mL
RSV B	N/A	B/3/2015 Isolate #2	200 GCE/mL	0.0131 $TCID_{50}$/mL

Example 9: SARS-CoV-2 Fast PCR Assay

[0223] Raw saliva samples were heated in a 95°C water bath for 30 minutes and subsequently allowed to equilibrate to room temperature. Each heat-treated sample was vortexed at maximum speed for 10 seconds or until the sample appeared homogenous. 100 µL of each heated treated saliva sample was transferred to individual wells of a 96-well plate having 100 µL of a TBE-T mix prepared therein. The TBE-T mix included 50 µL TBE buffer and 50 µL Tween-20 detergent. Each well was mixed by pipetting gently. Any storage prior to RT-PCR was done at 4°C or on ice for up to 2 hours.

[0224] The components in Table 30 were combined to make the RT-PCR Reaction Mix for the total number of reactions, plus 10% overage (with the Multiplex Reagents and Control reagents obtained from Thermo Fisher Catalog Nos. A47701 and 956125, respectively.

Table 30. RT-qPCR Reaction Mix

Component	Volume / reaction
TaqPath 1-Step Multiplex Master Mix, CG (4X)	2.5 µL
TaqMan SARS-CoV-2, Flu A, Flu B Multiplex Reagents	0.5 µL
Nuclease-free water	2.0 µL
Total Reaction Mix Volume	5.0 µL

[0225] The reaction mixes were vortexed for about 10–30 seconds) and centrifuged briefly. For each reaction, the components in Table 31, below, were combined in a MicroAmp Optical 384-Well Reaction Plate (0.2 mL/well):

Table 31. RT-qPCR Reactions

	Volume / reaction		
Component	RNA Sample	Pos. Control	Neg. Control
Reaction Mix (see Table 30)	5.0 µL	5.0 µL	5.0 µL
Prepared sample (saliva + TBE-T)	5.0 µL	—	—

Diluted TaqMan SARS-CoV-2, Flu A, Flu B, RNA Control	—	17.5 µL	—
Nuclease-free water	—	—	5.0 µL
Total Reaction Volume	10.00 µL	10.0 µL	10.0 µL

[0226] The plate was sealed with a MicroAmp Optical Adhesive Film and vortexed briefly to mix the contents. The plate was centrifuged briefly to collect the contents at the bottom of the wells. The plate was loaded into a 7500 Real-Time PCR Instrument and the protocol in Table 32 was run.

Table 32. RT-qPCR Protocol for Multi-Pathogen Multiplex Assay

Step	Stage	# of cycles	Temp.	Time
Reverse transcription	1	1	50°C	4 min
Polymerase activation	2	1	95°C	2 min
Amplification	3	40	95°C	1 sec
			60°C	20 sec

[0227] Appropriate analysis parameters were identified using the baseline threshold algorithm with manual threshold settings on Applied Biosystems qPCR instruments to calculate Ct values. With this algorithm, there were two primary analysis settings impacting Ct value—baseline and threshold. The baseline is set individually for each amplification curve and defines the region of baseline significant fluorescence signal detected, which can help normalize well-to-well variance in background noise during early cycles. Automatic baselining using a start cycle of 5 was initially used for the TaqCheck SARS-CoV-2 Fast PCR Assay.

[0228] Once the primary analysis settings were established, Ct cutoffs were defined for each target for both samples and controls. Cutoffs were evaluated using data generated with no template controls and other negative controls to exclude spurious amplification such as contamination introduced from the environment. Cutoffs were also assessed in context of data assessing the dynamic range of the assay. For example, an acceptable Ct cutoff excludes background contamination in the NTC while capturing true amplification within the verified dynamic range.

[0229] The first experiment determined the maximum level of background fluorescence signal and therefore the lowest that ΔRn thresholds could be set. This experiment consisted of running single-amplification wells from SARS-CoV-2 *in vitro* transcribed RNA ($1x10^7$ copies/well) and human universal human reference RNA (1 mg) in 4 corner wells and 4 wells in the center of a 384-well plate on 8 different QuantStudio 5 instruments.

[0230] The second experiment assessed variability of RNAse P Ct values and the real-world effects of inhibition on the overall strength of qPCR amplification and therefore how high ΔRn

thresholds could be set. This experiment was accomplished using frozen negative saliva samples (in triplicate) and spiking in dilutions of inactivated virus at 10,000 GCE/mL.

[0231] The final experiment established ΔRn and Ct cutoffs for assay targets. This was accomplished by running a total of 8, 384-well NTC plates at 3 different lab sites to determine the variability of RNase P background signal between different labs and establish a Ct cutoff settings for the assay. There is inherent variability between labs since human cells, though ubiquitous, exist at varying levels between different laboratories.

[0232] Results: once the data were collected, the ΔRn of representative present and absent samples for RNase P was plotted at various cycles to determine a preliminary Ct cutoff and threshold that separates the RNase P present from the RNase P absent samples. Preliminary experiments indicated that a Ct cutoff of 32 for RNase P for samples provided analytical sensitivity and guarded against false calls due to sample inadequacy. Based on these experiments a Ct cutoff of 35 was selected for RNase P in the No Template Control (NTC) and Positive Control (PC) to control for RNase P contamination as these controls should not contain human genomic material. Preliminary experiments further indicated that a Ct cutoff of 37 for SARS-CoV-2 provides analytical sensitivity while addressing low levels of SARS-CoV-2 contamination. It is important to note that high levels of contamination (such as from a cross-contamination event) are difficult to address by thresholds and Ct cutoffs—best practices should be implemented in the lab SOP to prevent contamination.

[0233] A summary of the thresholds and Ct cutoffs identified are provided in Table 33 below.

Table 33.

Target	Sample Type	Threshold (ΔRn)	Ct cutoff
RNase P	Sample	0.2	32
SARS-CoV-2	Sample, NTC	0.1	37
RNase P	PC, NTC	0.2	35
SARS-CoV-2	PC	0.1	37

[0234] Based on the foregoing, secondary analysis Ct cutoffs were applied for samples (as described in Table 34 below) and for NTC and PC (as described in Table 35 below).

Table 34.

Ct Value		Result
SARS-CoV-2 N and S genes (VIC)	RNase P (FAM)	
≤ 37	≤ 32	Present

≤ 37	> 32	Present
> 37	≤ 32	Absent
> 37	> 32	Re-Test

Table 35.

Control	Ct Value	
	SARS-CoV-2 N and S genes (VIC)	RNase P (FAM)
NTC	> 37	> 35
PC	≤ 37	> 35

[0235] To determine the analytical sensitivity, an experiment was performed to determine the genome copy number equivalents per mL (GCE/mL) where greater than 95% of the expected present samples were detected. Gamma irradiated virus was spiked into SARS-CoV-2 negative saliva samples. Samples were then prepared as described in TaqCheck™ SARS-CoV-2 Fast PCR Assay Quick Reference Guide and analyzed by RT-PCR. Data from a representative experiment, analyzed based on the determined threshold and Ct cutoffs contained herein, are presented in Table 36. Based on the results of the experiment, analytical sensitivity was established at 6,000 GCE/mL.

Table 36.

Copies (GCE/mL)	# Replicates	# Pos Samples Detected	% Pos
1000	92	46	50%
3320	96	87	91%
4000	300	192	64%
6000	80	79	99%
6680	96	93	97%
9000	60	60	100%
10000	280	274	98%
12000	60	60	100%
20000	161	161	100%

Example 10: Discrimination of variants of SARS-CoV-2 in biological samples

[0236] In some embodiments, one inherent advantage possessed by the disclosed primers and probes is the ability to discriminate between patient samples that contain the 'normal' or 'reference' version of SARS-nCoV-2, as exemplified in GenBank Accession No: MN908947.3, and patient samples infected by certain viral variants, particularly variants involving deletion of amino acid residue 69 and/or 70 of the Spike protein encoded by the S gene. The disclosed primers and probes can accordingly be used to quickly and cheaply determine whether certain

SARS-CoV-2 variants are potentially present in clinical samples during patient intake or triage. Samples that test positive for two out of three viral target sequences (N, S and ORF1ab) are initially classified as positive and selected for further assessment using more extensive and expensive confirmatory methods such as sequencing of the viral genome. As reported in the literature, such primers and probes have been widely used by health facilities in the United Kingdom to obtain an initial indication of whether the B.1.1.7 variant is present in patients.

[0237] *See, e.g., Public Health England, Technical Briefing 1, Investigation of Novel SARS-COV-2 Variant, Variant of Concern 202012/01,* available at https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/959438/Technical_Briefing_VOC_SH_NJL2_SH2.pdf.

[0238] As an example, samples collected from humans via nasopharyngeal swab, nasopharyngeal aspirate, or bronchoalveolar lavage are tested to determine whether certain variants of SARS-CoV-2 are potentially present. Viral DNA from patient samples is isolated using the MagMAX Viral/Pathogen Nucleic Acid Isolation Kit (sold by Thermo Fisher Scientific under Cat. No. A42356) in accordance with the instructions provided therewith.

[0239] The samples are then subjected to multiplex amplification using the exemplary primers and probes specified below and using the master mix and other general components from the TaqPath COVID-19 Combo Kit (Thermo Fisher Scientific, Catalog No. A47814) in accordance with the protocol supplied therewith. Amplification is performed on a QuantStudio 7500 (Thermo Fisher Scientific) and amplification of the N, S and ORF1ab target sequences monitored using the VIC, ABY and FAM probe labels, respectively, while the positive control (MS2) was monitored using a JUN probe label.

Table 37.

	FOR primer	REV primer	Probe
ORF1ab	SEQ ID NO: 160	SEQ ID NO: 468	SEQ ID NO: 1049
S	SEQ ID NO: 100	SEQ ID NO: 337	SEQ ID NO: 864
N	SEQ ID NO: 211	SEQ ID NO: 510(mod)	SEQ ID NO: 833

[0240] The resulting data are analyzed using the COVID Interpretive Software released by Thermo Fisher Scientific. For each plate, the control reactions are confirmed to perform as expected (*i.e.,* the no template control have an undetermined C_t value and the positive control has a C_t value less than or equal to 30).

[0241] The Ct cutoff for viral samples is set to 37. If the C_t value for each of the N, S and ORF1ab target sequences is determined, the sample called as positive for a target sequence where the Ct value for that target sequence is equal to, or less than, 37. In several samples, the results were positive for the N and ORF1ab gene target sequences but negative for the S gene target sequence.

[0242] Samples that test positive for two or more SARS-CoV-2 target sequences are classified as having a valid result; those only showing positive for two of the three SARS-CoV-2 target sequences are recommended for further testing by the health authorities. These can typically, but not necessarily, include samples that test positive for both the N and ORF1ab gene target sequences while testing negative for the S gene target sequence.

Conclusion

[0243] The present disclosure may be embodied in other specific forms without departing from its spirit or essential characteristics. The described embodiments are to be considered in all respects only as illustrative and not restrictive. The scope of the invention is, therefore, indicated by the appended claims rather than by the foregoing description. While certain embodiments and details have been included herein and in the attached disclosure for purposes of illustrating embodiments of the present disclosure, it will be apparent to those skilled in the art that various changes in the methods, products, devices, and apparatuses disclosed herein may be made without departing from the scope of the disclosure or of the invention. Thus, while various aspects and embodiments have been disclosed herein, other aspects and embodiments are contemplated. All changes that come within the meaning and range of equivalency of the claims are to be embraced within their scope.

[0244] The subsequent items are a list of preferred embodiments:

1. A method for detecting SARS-CoV-2 in a nucleic acid sample, comprising:
   (a) creating a reaction mixture containing the nucleic acid sample, a forward primer, and a reverse primer; and
   (b) subjecting the reaction mixture to reaction conditions suitable to perform a polymerase chain reaction (PCR).
2. The method of item 1, further including generating one or more amplicons via PCR.

3. The method of item 1 or item 2, wherein the reaction mixture further includes a probe containing a fluorescent reporter and a corresponding quencher.
4. The method of any one of items 1–3, further including monitoring the fluorescence produced during the PCR.
5. The method of any preceding item, further including determining the amount of nucleic acid present in the sample.
6. The method of any preceding item, wherein the forward primer and the reverse primer bind to a region of the coronavirus genome wherein the homology of the region between SARS-CoV-2 and bat-SL-CoVZC45 is less than 50%, 20%, 10% or 5%.
7. The method of item 6, wherein the region is within the ORF1ab gene, the S protein gene, or the N protein gene of the SARS-CoV-2 genome.
8. The method of any preceding item, wherein the forward primers are selected from SEQ ID NO:4 – SEQ ID NO:251.
9. The method of any preceding item, where in the reverse primers are selected from SEQ ID NO:267 – SEQ ID NO:504.
10. The method of item any preceding item, wherein the probe sequence is selected from SEQ ID NO:520 – SEQ ID NO:1295.
11. The method of any one of items 3-10, wherein the probe is labeled at the 5' end with a dye selected from 6FAM, ABY, VIC, JUN, and FAM.
12. The method of item 11, wherein the probe is labeled at the 3' end with a quencher selected from QSY, BHQ (Black Hole Quencher), and DFQ (Dark Fluorescent Quencher).
13. The method of any preceding item, wherein a positive control and a negative control are analyzed in conjunction with the sample.
14. The method of item 13, wherein a nucleic acid template for the positive control is a synthetic plasmid comprising sequences from the SARS-CoV-2 ORF1ab gene, the SARS-CoV-2 S protein gene, the SARS-CoV-2 N protein gene, and/or a human RNase P gene.
15. A composition for detecting the presence of SARS-CoV-2 from a nucleic acid sample, comprising a nucleic acid primer and/or probe containing a nucleic acid sequence of a target region, the nucleic acid primer and/or probe comprising a primer and/or probe within SEQ ID NO:4 – SEQ ID NO:251. SEQ ID NO:267 – SEQ ID NO:504, and SEQ ID NO:520 – SEQ ID NO:1295.

16. The composition of item 15, wherein the nucleic acid primer is a first forward primer configured to hybridize to one end of a first target sequence within a first target region in the SARS-CoV-2 viral RNA genome, or to a complement of the first target sequence.
17. The composition of item 15 or 16, wherein the nucleic acid sequence of the target region is SEQ ID NO:1.
18. The composition of item 15 or 16, wherein the nucleic acid sequence of the target region is SEQ ID NO:2.
19. The composition of item 15 or 16, wherein the nucleic acid sequence of the target region is SEQ ID NO:3.
20. The composition of any one of items 15–19, further including a first reverse primer configured to hybridize to the other end of the first target sequence or its complement.
21. The composition of any one of items 15–20, further including the nucleic acid sample, a polymerase, a buffer, and dNTPs.
22. The composition of any one of items 15–21, further including a first probe containing a detectable label.
23. The composition of item 22, wherein the detectable label is a fluorescent label and the first probe further includes a quencher that quenches the fluorescent label.
24. The composition of item 22 or item 23, wherein the first probe is configured to hybridize to a first target subsequence that is complementary or identical to at least 10 contiguous nucleotides within the first target sequence, a DNA copy thereof, or to a complement of the first target sequence or its DNA copy.
25. A composition for amplifying one or more target sequences in the SARS-CoV-2 genome, comprising: a first forward primer and a first reverse primer configured to amplify a first target sequence present in a first target region of the SARS-CoV-2 genome, wherein the first target sequence includes at least 10 contiguous nucleotides of the first target region, the first target region having less than 50%, 40%, 30%, 20%, or 10% identity with an analogous region in bat-SL-CoVZC45.
26. The composition of item 25, wherein the first forward primer and the first reverse primer are configured to hybridize to different ends of the first target sequence, a DNA copy thereof, or their respective complements, and form an amplicon therebetween.
27. The composition of item 25 or item 26, wherein the nucleic acid sequence of the first target region is SEQ ID NO:1.
28. The composition of item 25 or item 26, wherein the nucleic acid sequence of the first target region is SEQ ID NO:2.

29. The composition of item 25 or item 26, wherein the nucleic acid sequence of the first target region is SEQ ID NO:3.
30. The composition of any one of items 25–29, further including a nucleic acid sample, a polymerase, a buffer, and dNTPs.
31. The composition of any one of items 25–30, further including a first probe containing a detectable label.
32. The composition of item 31, wherein the detectable label is a fluorescent label and the first probe further includes a quencher that quenches the fluorescent label.
33. The composition of item 31 or 32, wherein the first probe is configured to hybridize to a first target subsequence that is complementary or identical to at least 10 contiguous nucleotides of the first target sequence, a DNA copy thereof, or their respective complements.
34. The composition of item 33, further including a second forward primer and a second reverse primer configured to amplify a second target sequence within a second target region of the SARS-CoV-2 genome.
35. The composition of item 34, wherein the second forward primer and the second reverse primer are configured to bind to different ends of the second target sequence or to a cDNA complement thereof.
36. The composition of item 34 or 35, wherein the nucleic acid sequence of the first target region and the second target region are different and are selected from SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3.
37. The composition of item 36, further including a third forward primer and a third reverse primer configured to amplify a third target sequence within a third target region of the SARS-CoV-2 genome.
38. The composition of item 37, wherein the third forward primer and the third reverse primer are configured to bind to different ends of the third target sequence or to a DNA copy or DNA complement thereof.
39. The composition of item 38, wherein the nucleic acid sequence of the first target region, the second target region, and the third target region are different and are selected from SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3.
40. The composition of any one of items 26-39, wherein the primer sequences specific for the first target sequence are selected from SEQ ID NOs: 4, 320, 34, 423, 160 and 468.
41. The composition of any one of items 34-40, wherein the primer sequences specific for the second target sequence are selected from SEQ ID NOs: 5, 441, 100 and 337.

42. The composition of any one of items 37-41, wherein the primer sequences specific for the third target sequence are selected from SEQ ID NOs : 248, 487, 211 and 501.
43. A composition useful as an amplification control in a reaction to detect SARS-CoV-2 nucleic acid, Influenza (Flu) type A and/or Influenza (Flu) type B nucleic acid, and/or Respiratory Syncytia Virus (RSV) nucleic acid in a sample, comprising a linear or circular nucleic acid molecule including, in any order, at least one target sequence derived from the N gene of SARS-CoV-2, at least one further target sequence derived from the S gene of SARS-CoV-2, and optionally at least one target sequence derived from the ORF1ab gene of SARS-CoV-2.
44. A kit for detecting SARS-CoV-2 nucleic acid, Influenza (Flu) type A and/or Influenza (Flu) type B nucleic acid, and/or Respiratory Syncytia Virus (RSV) nucleic acid in a sample, comprising a composition of any one of items 15–43, or any combinations thereof.
45. The kit of item 44, further including a PCR master mix.
46. The kit of item 45, wherein the master mix is TaqMan Fast Virus 1-Step Master Mix or TaqPath 1-Step RT-qPCR Master Mix, CG.
47. The kit of any one of items 44–46, wherein at least one of the components is dried or freeze dried.
48. The kit of any of items 44–47, further including an array of qPCR assays, each qPCR assay situated in a different locus of the array.
49. The kit of item 48, wherein the different locus includes a well, channel, groove, cavity, site, or feature formed on a surface of the array.
50. A method of detecting SARS-CoV-2 viral nucleic acid present in a sample, comprising:
    (a) providing a composition according to any one of items 15–43;
    (b) forming a reaction volume by contacting the composition, in any order or combination, with a polymerase, dNTPs, and a nucleic acid sample obtained from bodily tissue taken from an organism; and
    (c) forming one or more amplification products containing amplified SARS-CoV-2 sequences in the reaction volume, wherein the forming includes subjecting the reaction volume to amplification conditions suitable to amplify target SARS-CoV-2 sequences from SARS-CoV-2 nucleic acid present in the nucleic acid sample prior to amplification.
51. The method of item 50, further including detecting at least one of the amplification products during or after the forming step.

52. The method of item 50 or 51, further including diagnosing a SARS-CoV-2 infection in the organism.
53. The method of any one of items 50–52, wherein the organism is a human subject and the nucleic acid sample is derived from SARS-CoV-2.
54. The method of any one of items 50–53, wherein the forming includes amplifying at least three different and specific SARS-CoV-2 target sequences from the nucleic acid sample.
55. The method of item 54, wherein the at least three different three different SARS-CoV-2 target sequences include one target sequence derived from the N gene, one target sequence derived from the S gene, and one target sequence derived from the ORF1ab gene.
56. The method of item 13 or any one of items 50 to 54, wherein the positive control is an exogenous RNA sequence or an endogenous DNA or RNA sequence.
57. The method of item 56, wherein the exogenous RNA sequence is an MS2 bacteriophage sequence and wherein the endogenous DNA or RNA sequence is a human RNase P sequence.
58. The method of item 56 or 57, further comprising a second positive control selected from an exogenous RNA sequence and an endogenous nucleic acid sequence.
59. The method of any one of items 1–14 or 50–58, wherein the method further includes detecting a target sequence derived from the influenza type A (Flu A) and/or influenza type B (Flu B) virus in the nucleic acid sample.
60. The method of item 59, wherein the detecting comprises the use of a forward primer selected from SEQ ID NO:252, SEQ ID NO:253, or SEQ ID NO:257; a reverse primer selected from SEQ ID NO:505 and SEQ ID NO:506; and/or a probe selected from SEQ ID NO:1296 and SEQ ID NO:1297.
61. The method of any one of items 1–14 or 50–60, wherein detection of SARS-CoV-2 and/or Flu A and/or Flu B in the nucleic acid sample is detected down to at least a 10 genomic copy equivalent per reaction (GCE/rxn).
62. The method of any one of items 1–14 or 50–60, wherein detection of SARS-CoV-2 and/or Flu A and/or Flu B in the nucleic acid sample is detected over a linear dynamic range (LDR) of detection from 10^7 to 10 GCE/rxn.
63. The method of any one of items 1–14 or 50–60, wherein detection of SARS-CoV-2 in the nucleic acid sample is detected down to 1–10 copies/μL per reaction.
64. The method of any one of items 1–14 or 50–60, wherein detection of SARS-CoV-2 in the nucleic acid sample is detected over a linear dynamic range (LDR) of at least 5 logs.

65. A composition for detecting the presence of SARS-CoV-2 and Influenza type A (Flu A) and/or Influenza type B (Flu B) in a nucleic acid sample, comprising at least two pairs of nucleic acid primers, each pair of nucleic acid primers having a forward primer and a reverse primer selected, respectively, from SEQ ID NO:4 – SEQ ID NO:257 and SEQ ID NO:267 – SEQ ID NO:510, and, optionally, at least two nucleic acid probes containing a nucleic acid sequence selected from any of SEQ ID NO:520 – SEQ ID NO:2533.
66. The method, kit or composition of any one of the preceding items, wherein the probe is a FAM-labeled probe directed to the ORF1ab gene of SARS-CoV-2.
67. The method, kit or composition of any one of the preceding items, wherein the probe is a VIC-labeled probe directed to the N protein gene of SARS-CoV-2.
68. The method, kit or composition of any one of the preceding items 1, wherein the probe is an ABY-labeled probe directed to the S protein gene of SARS-CoV-2.
69. The method, kit or composition of any one of the preceding items, wherein the positive control is an MS2 qPCR assay comprising a JUN-labeled probe directed to a portion of the MS2 nucleic acid present in the MS2 qPCR assay.
70. A method for the detection of SARS-CoV-2 in a nucleic acid sample, comprising:
(a) creating a reaction mixture containing the nucleic acid sample, a forward primer, and a reverse primer; and
(b) subjecting the reaction mixture to reaction conditions suitable to perform a loop-mediated isothermal amplification (LAMP).
71. The method of any one of items 1–14, 50–64, and 66–70, wherein the method comprises a point-of service (POS) system.
72. The method of any one of items 1–14, 50–64, and 66–70, wherein the nucleic acid sample is collected at a point of care (POC) location, and/or is analyzed in a device at the POC location.
73. The method of item 71, wherein the device at the POC location is configured to analyze a small-volume clinical sample in a short period of time, such as less than 1–2 hours.
74. The method of item 71 or item 72, wherein the method is performed on the POS system at the POC location.
75. The method of item 71 or item 72, wherein the nucleic acid sample is obtained at the POC location.
76. The method of item 71 or item 72, wherein the method is used for analyzing a clinical sample at the POC location.

77. The method of item 71 or item 72, wherein the POS method includes performing a plurality of assays on a single small volume clinical sample, or on aliquots thereof.
78. The method of item 71, wherein the POS system is implemented at a POS location, and wherein the method is performed in a short period of time.
79. The method of item 71, wherein the methods are implemented at the POS system at the POS location, wherein the methods are for performing a plurality of assays on a single small volume clinical sample, or on aliquots thereof, and may be performed in a short period of time.
80. The method of item 78 or item 79, wherein the short period of time is less than 24 hours.
81. The method, kit or composition of any one of the preceding items, wherein the PCR is a reverse transcription PCR (RT-PCR).
82. The method, kit or composition of any one of the preceding items, wherein a forward RNase P primer comprises SEQ ID NO:2552, a reverse RNase P primer comprises SEQ ID NO:2553, and/or an RNase P probe is selected from SEQ ID NO:2554 – SEQ ID NO:2556.
83. The method of any one of items 1–14 or 50–64 or 66-82, wherein the method further includes detection of a Respiratory Syncytial Virus (RSV) specific target within the nucleic acid sample.
84. The method of item 83, wherein detection of RSV comprises detecting RSV type A and/or RSV type B using a forward primer selected from SEQ ID NO:254 and SEQ ID NO:255, a reverse primer selected from SEQ ID NO:507 and SEQ ID NO:508, and/or a probe selected from SEQ ID NO:1298 and SEQ ID NO:1299.
85. The method of any one of items 1–14 or 50–64 or 66-84, wherein detection of SARS-CoV-2, Flu A and/or Flu B, and/or RSV A and/or RSV B in the nucleic acid sample is detected down to at least a 10 genomic copy equivalent per reaction (GCE/rxn).
86. The method of any one of items 1–14 or 50–64 or 66-84, wherein detection of SARS-CoV-2, Flu A and/or Flu B, and/or RSV A and/or RSV B in the nucleic acid sample is detected over a linear dynamic range of detection from 10^7 to 10 GCE/rxn.
87. The method of any one of items 1–14 or 50–64 or 66-84, wherein the method further includes detection of influenza type A (Flu A) virus, influenza type B (Flu B) virus, Respiratory Syncytial Virus type A (RSV A), and/or Respiratory Syncytial Virus type B (RSV B) in the nucleic acid sample.

88. The method of item 87, wherein the SARS-CoV-2 probes comprise a VIC dye and QSY quencher, the Flu A/B probes comprise FAM dye and QSY quencher, and the RSV A/B probes comprise an ABY dye and QSY quencher.
89. A method for detecting SARS-CoV-2 as disclosed herein.
90. The method of any one of items 1–14, 50–64, or 66–89, wherein the sample comprises a saliva sample.
91. The method of item 89 or item 90, wherein the method does not include a step for purifying or extracting a nucleic-acid-containing portion away from the sample.
92. The method of any one of items 89–91, wherein the detection of SARS-CoV-2 within the sample is performed via a nucleic acid amplification reaction utilizing the unpurified sample as a probative template.
93. The method of item 92, further comprising heating the sample for a period of time sufficient to inactivate nucleases within the sample and/or to rupture eukaryotic cells, denature viral capsids, and/or disrupt a membrane portion of enveloped virions therein.
94. The method of any one of items 89–93, further comprising heating the unpurified sample to a temperature at or above about 80°C, preferably at or above about 90°C, more preferably at or above about 95°C.
95. The method of item 94, wherein the unpurified sample is heated for at least 5, 10, 15, 20, 25, 30, 35, or 40 minutes or for any range of time formed by an upper and lower bound selected therefrom.
96. The method of any one of items 90–95, further comprising combining the sample with a lysis buffer.
97. The method of any one of items 93–96, further comprising mixing the heat-treated sample prior to and/or after combining the heat-treated sample with the lysis buffer.
98. The method of item 96 or 97, wherein the lysis buffer comprises a nucleic-acid-amenable buffer and a detergent and/or emulsifier.
99. The method of any one of items 96–98, wherein the lysis buffer comprises a combination of TBE buffer and a polysorbate-type nonionic surfactant, such as Tween-20.
100. The method of any one of items 89–99, wherein the sample comprises a pooled-subject sample.
101. The method of any one of items 89–100, wherein detecting SARS-CoV-2 within the sample occurs in less than about 3 hours from the time the sample is received, preferably less than about 2 hours.

102. A method for the detection of the SARS-CoV-2 coronavirus in a nucleic acid sample comprising:
(a) heating a sample for 15–45 minutes, preferably about 30 minutes, at 95°C;
(b) mixing the heat-treated sample with a lysis solution to form a volume of probative template;
(c) creating a nucleic acid amplification reaction mixture comprising at least a portion of the volume of probative template, one or more primers specific and/or diagnostic for SARS-CoV-2, and a nucleic acid polymerase; and
(d) subjecting the nucleic acid amplification reaction mixture to conditions suitable to generate SARS-CoV-2-specific amplicons if SARS-CoV-2 nucleic acid is present in the sample.

103. The method of item 102, further comprising receiving a sample.

104. The method of item 103, wherein receiving the sample comprises receiving a sample collection device or other container comprising the sample.

105. The method of item 104, wherein the sample collection device is a sealable tube.

106. The method of any one of items 103–105, wherein the sample is received following self-collection of the sample by the subject.

107. The method of any one of items 103–106, wherein receiving the sample comprises receiving a raw saliva sample.

108. The method of any one of items 102–107, further comprising vortexing the heat-treated sample.

109. The method of any one of items 102–108, further comprising detecting the amplicons, or one or more detectable labels associated with generation of the amplicons, while subjecting the nucleic acid amplification reaction mixture to the conditions suitable for generating the amplicons.

110. The method of any one of items 102–108, further comprising detecting the amplicons, or one or more detectable labels associated with generation of the amplicons, after the nucleic acid amplification reaction mixture is subjected to the conditions suitable for generating the amplicons.

111. The method of any one of items 102–110, further comprising equilibrating the heat-treated sample to room temperature prior to mixing the heat-treated sample with the lysis solution.

112. The method of any one of the preceding items, wherein the sample is received following self-collection by the sample provider.

113. The method of any one of the preceding items, wherein the sample is received following collection by an individual other than the sample provider.
114. The method of any one of the preceding items, wherein one or more of the method steps are performed using a sample collection device.
115. The method of item 114, wherein at least the steps of receiving and heating are performed using the sample collection device.
116. The method of any one of the preceding items, wherein the method is used for asymptomatic testing and/or high-frequency or widespread screening.
117. A composition for use in methods of detecting viral nucleic acid, the composition comprising a heat-treated sample.
118. The composition of item 117, wherein the heat-treated sample comprises heat-treated raw saliva.
119. The composition of item 117 or item 118, further comprising a buffer.
120. The composition of item 119, wherein the buffer comprises TBE.
121. The composition of any one of items 117–120, further comprising a detergent and/or emulsifier.
122. The composition of item 121, wherein the detergent and/or emulsifier comprises a polysorbate-type nonionic surfactant.
123. The composition of any one of items 117–122, further comprising one or more PCR reagents.
124. The composition of item 123, wherein the one or more PCR reagents comprise one or more primers or probes for amplifying specific viral nucleic acid sequences as described herein.
125. The composition of item 123 or item 124, wherein the one or more PCR reagents comprise a PCR master mix or components thereof.
126. The method, composition or kit of any one of the preceding items, wherein the nucleic acid sample is derived from a non-human animal.
127. The method, composition or kit of any one of the preceding items, wherein the nucleic acid sample is derived from a mammal.
128. The method, composition or kit of any one of item 127, wherein the nucleic acid sample is derived from a mink, cat, dog, ferret, hamster, bat, primate, such as Rhesus macaques, cynomolgus macaques, grivets, and common marmosets, zoo animal, laboratory animal or farm animal.

129. The method, composition or kit of any one of the preceding items, wherein the reverse and forward primer sequences specific for the first target sequence are selected from SEQ ID NOs: 248 and 487, or 211 and 501.

130. The method, composition or kit of any one of the preceding items wherein the reverse and forward primer sequences specific for the second target sequence are selected from SEQ ID NOs: 5 and 441, or 100 and 337.

131. The method, composition or kit of any one of the preceding items, wherein the reverse and forward primer sequences specific for the third target sequence are selected from SEQ ID NOs: 4 and 320, or 34 and 423, or 160 and 468.

132. The method, composition or kit of any one of the preceding items, wherein the reverse and forward primer sequences specific for the first, second and/or third target sequence are:

SEQ ID NOs: 4 and 320, SEQ ID NOs: 5 and 441 and/or SEQ ID NOs: 248 and 487;

SEQ ID NOs: 34 and 423, SEQ ID NOs: 5 and 441 and/or SEQ ID NOs: 248 and 487;

SEQ ID NOs: 160 and 468, SEQ ID NOs: 5 and 441 and/or SEQ ID NOs: 248 and 487;

SEQ ID NOs: 4 and 320, SEQ ID NOs: 100 and 337 and/or SEQ ID NOs: 248 and 487;

SEQ ID NOs: 34 and 423, SEQ ID NOs: 100 and 337 and/or SEQ ID NOs: 248 and 487;

SEQ ID NOs: 160 and 468, SEQ ID NOs: 100 and 337 and SEQ ID NOs: 248 and 487

SEQ ID NOs: 4 and 320, SEQ ID NOs: 5 and 441 and SEQ ID NOs: 211 and 501;

SEQ ID NOs: 34 and 423, SEQ ID NOs: 5 and 441 and SEQ ID NOs: 211 and 501;

SEQ ID NOs: 160 and 468, SEQ ID NOs: 5 and 441 and SEQ ID NOs: 211 and 501;

SEQ ID NOs: 4 and 320, SEQ ID NOs: 100 and 337 and SEQ ID NOs: 211 and 501;

SEQ ID NOs: 34 and 423, SEQ ID NOs: 100 and 337 and SEQ ID NOs: 211 and 501; or

SEQ ID NOs 160 and 468, SEQ ID NOs: 100 and 337 and SEQ ID NOs: 211 and 501.

133. The method, composition or kit of any one of the preceding items, wherein the probe is selected from the group consisting of SEQ ID NO: 565, 599, 971, 930, 1160, 1106, 1203, 1049, 864 and/or 833.

134. The method, composition or kit of any one of the preceding items, wherein any one of the reverse and forward primer sequences specific for the first, second or third target sequence is SEQ ID NO: 248.
135. The method, composition or kit of any one of the preceding items, wherein any one of the reverse and forward primer sequences specific for the first, second or third target sequence is SEQ ID NO: 487.
136. The method, composition or kit of any one of the preceding items, wherein any one of the reverse and forward primer sequences specific for the first, second or third target sequence is SEQ ID NO: 211.
137. The method, composition or kit of any one of the preceding items, wherein any one of the reverse and forward primer sequences specific for the first, second or third target sequence is SEQ ID NO: 501.
138. The method, composition or kit of any one of the preceding items, wherein any one of the reverse and forward primer sequences specific for the first, second or third target sequence is SEQ ID NO: 5.
139. The method, composition or kit of any one of the preceding items, wherein any one of the reverse and forward primer sequences specific for the first, second or third target sequence is SEQ ID NO: 441.
140. The method, composition or kit of any one of the preceding items, wherein any one of the reverse and forward primer sequences specific for the first, second or third target sequence is SEQ ID NO: 100.
141. The method, composition or kit of any one of the preceding items, wherein any one of the reverse and forward primer sequences specific for the first, second or third target sequence is SEQ ID NO: 337.
142. The method, composition or kit of any one of the preceding items, wherein any one of the reverse and forward primer sequences specific for the first, second or third target sequence is SEQ ID NO: 160.
143. The method, composition or kit of any one of the preceding items, wherein any one of the reverse and forward primer sequences specific for the first, second or third target sequence is SEQ ID NO: 468.
144. The method, composition or kit of any one of the preceding items, wherein any one of the reverse and forward primer sequences specific for the first, second or third target sequence is SEQ ID NO: 4.

145. The method, composition or kit of any one of the preceding items, wherein the probe is SEQ ID NO: 565.
146. The method, composition or kit of any one of the preceding items, wherein the probe is SEQ ID NO: 599.
147. The method, composition or kit of any one of the preceding items, wherein the probe is SEQ ID NO: 971.
148. The method, composition or kit of any one of the preceding items, wherein the probe is SEQ ID NO: 930.
149. The method, composition or kit of any one of the preceding items, wherein the probe is SEQ ID NO: 1160.
150. The method, composition or kit of any one of the preceding items, wherein the probe is SEQ ID NO: 1106.
151. The method, composition or kit of any one of the preceding items, wherein the probe is SEQ ID NO: 1203.
152. The method, composition or kit of any one of the preceding items, wherein the probe is SEQ ID NO: 1049.
153. The method, composition or kit of any one of the preceding items, wherein the probe is SEQ ID NO: 864.
154. The method, composition or kit of any one of the preceding items, wherein the probe is SEQ ID NO: 833.
155. The method, composition or kit of any one of the preceding items, wherein any one of the reverse and forward primer sequences specific for the first, second or third target sequence is SEQ ID NO: 320.
156. The method, composition or kit of any one of the preceding items, wherein any one of the reverse and forward primer sequences specific for the first, second or third target sequence is SEQ ID NO: 34.
157. The method, composition or kit of any one of the preceding items, wherein any one of the reverse and forward primer sequences specific for the first, second or third target sequence is SEQ ID NO: 423.
158. The method, composition or kit of any one of the preceding items, comprising a first forward primer of SEQ ID NO: 160 and a first reverse primer of SEQ ID NO: 468.
159. The method, composition or kit of item 134, further comprising a probe of SEQ ID NO: 1049.

160. The method, composition or kit of any one of the preceding items, comprising a second forward primer of SEQ ID NO: 100 and a second reverse primer of SEQ ID NO: 337.

161. The method, composition or kit of item 136, further comprising a probe of SEQ ID NO: 864.

162. The method, composition or kit of any one of the preceding items, comprising a third forward primer of SEQ ID NO: 211 and a third reverse primer of SEQ ID NO: 501 and/or 510.

163. The method, composition or kit of item 138, further comprising a probe of SEQ ID NO: 833.

164. The method, composition or kit of any one of the preceding items useful for amplifying and/or detecting a region of the ORF1ab gene of SARS-CoV-2, comprising: a forward primer of SEQ ID NO: 160, a reverse primer of SEQ ID NO: 468, and a probe of SEQ ID NO: 1049.

165. The method, composition or kit of any one of the preceding items useful for amplifying and/or detecting a region of the S gene of SARS-CoV-2, comprising: a forward primer of SEQ ID NO: 100, a reverse primer of SEQ ID NO: 337, and a probe of SEQ ID NO: 864.

166. The method, composition or kit of any one of the preceding items useful for amplifying and/or detecting a region of the N gene of SARS-CoV-2, comprising: a forward primer of SEQ ID NO: 211, a reverse primer of SEQ ID NO: 501, and a probe of SEQ ID NO: 833.

167. The method, composition or kit of any one of the preceding items useful for amplifying and/or detecting a region of the N gene of SARS-CoV-2, comprising: a forward primer of SEQ ID NO: 211, a reverse primer of SEQ ID NO: 510, and a probe of SEQ ID NO: 833.

168. The method, composition or kit of any one of the preceding items useful for multiplex detection of target sequences derived from the S gene and the N gene of SARS-CoV-2, comprising:

(i) a first forward primer of SEQ ID NO: 211, a first reverse primer of SEQ ID NO: 501, and a first probe of SEQ ID NO: 833; and

(ii) a second forward primer of SEQ ID NO: 100, a second reverse primer of SEQ ID NO: 337, and a second probe of SEQ ID NO: 864.

169. The method, composition or kit of any one of the preceding items useful for multiplex detection of target sequences derived from the S gene and the N gene of SARS-CoV-2, comprising:

(i) a first forward primer of SEQ ID NO: 211, a first reverse primer of SEQ ID NO: 510, and a first probe of SEQ ID NO: 833; and

(ii) a second forward primer of SEQ ID NO: 100, a second reverse primer of SEQ ID NO: 337, and a second probe of SEQ ID NO: 864.

170. The method, composition or kit of any one of the preceding items useful for multiplex detection of target sequences derived from the S gene, N gene and ORF1ab genes of SARS-CoV-2, comprising:

(i) a first forward primer of SEQ ID NO: 160, a first reverse primer of SEQ ID NO: 468, and a first probe of SEQ ID NO: 1049;

(ii) a second forward primer of SEQ ID NO: 100, a second reverse primer of SEQ ID NO: 337, and a second probe of SEQ ID NO: 864; and

(iii) a third forward primer of SEQ ID NO: 211, a third reverse primer of SEQ ID NO: 501, and a third probe of SEQ ID NO: 833.

171. The method, composition or kit of any one of the preceding items useful for multiplex detection of target sequences derived from the S gene, N gene and ORF1ab genes of SARS-CoV-2, comprising:

(i) a first forward primer of SEQ ID NO: 160, a first reverse primer of SEQ ID NO: 468, and a first probe of SEQ ID NO: 1049;

(ii) a second forward primer of SEQ ID NO: 100, a second reverse primer of SEQ ID NO: 337, and a second probe of SEQ ID NO: 864; and

(iii) a third forward primer of SEQ ID NO: 211, a third reverse primer of SEQ ID NO: 510, and a third probe of SEQ ID NO: 833.

172. The composition or kit of any one of the preceding items, further comprising a primer selected from SEQ ID NOs:252, 253, or 257.

173. The composition or kit of any one of the preceding items, further comprising a primer selected from SEQ ID NOs: 505 and 506.

174. The composition or kit of any one of the preceding items, further comprising a probe selected from SEQ ID NOs:1296 and 1297.

175. The composition or kit of any one of the preceding items, further comprising a primer selected from SEQ ID NOs:254 and 255.

176. The composition or kit of any one of the preceding items, further comprising a primer selected from SEQ ID NOs:507 and:508.

177. The composition or kit of any one of the preceding items, further comprising an oligonucleotide selected from SEQ ID NOs:1298 and 1299.

178. The composition or kit of any one of the preceding items, further comprising an oligonucleotide selected from SEQ ID NOs:2552 and 2553.

179. The composition or kit of any one of the preceding items, further comprising an oligonucleotide selected from SEQ ID NOs:2554, 2555 and 2556.

180. The composition or kit of any one of the preceding items, further comprising one or more oligonucleotides selected from SEQ ID NO:256, 509 and 1300.

181. The composition or kit of any one of the preceding items, wherein any one or more of the primers and probes includes a fluorescent dye label.

182. The composition or kit of any one of the preceding items, wherein any one or more of the primers and probes includes a fluorescent dye label selected from the group consisting of: VIC, ABY, FAM and JUN.

183. A method for using the composition or kit of any one of the preceding items, comprising amplifying a target sequence using the composition or kit; and detecting the target sequence.

184. The method of item 183, further including determining whether a biological sample includes DNA or RNA from a virus.

185. The method of item 184, further including diagnosing a viral infection in the subject from which the biological sample was derived.

186. The method of item 185, further including diagnosing a specific viral infection in the subject from which the biological sample was derived.

187. The method of items any of items 183-186, further including ruling out a specific viral infection in the subject from which the biological sample was derived.

188. A method for the detection of the SARS-CoV-2 coronavirus in a nucleic acid sample comprising:
(a) creating a reaction mixture containing the sample, a forward primer and a reverse primer; and
(b) subjecting the reaction mixture to reaction conditions suitable to perform a polymerase chain reaction (PCR).

189. The method of item 188, further including generating one or more amplicons via PCR.

190. The method of item 188 or 189, wherein the reaction mixture further includes a probe containing a fluorescent reporter and a corresponding quencher.

191. The method of any of items 188-190, further including monitoring and/or detecting the fluorescence produced during the PCR.

192. The method of item 191, further including determining the amount of nucleic acid present in the sample.

193. The method of item 188, wherein the forward primer and the reverse primer bind to a region of the coronavirus genome wherein the homology between the SARS-CoV-2 coronavirus and the bat-SL-CoVZC45 coronavirus is less than 50%, 20%, 10% or 5%.

194. The method of item 193, wherein the region of the coronavirus having less than 50% homology between the SARS-CoV-2 coronavirus and the bat-SL-CoVZC45 coronavirus is within the orf1ab gene, the S protein gene or the N protein gene of the coronavirus genome.

195. The method of item 193 or 194, wherein the forward primers are selected from SEQ ID NO. 4-SEQ ID NO. 251.

196. The method of item 193 or 194, where in the reverse primers are selected from SEQ ID NO. 267-SEQ ID NO. 504.

197. The method of item 193 or 194, wherein the probe sequence is selected from SEQ ID NO. 520-SEQ ID NO. 1295.

198. The method of item 197, wherein the probe is labeled at the 5' end with a dye selected from 6FAM, ABY, VIC, JUN and FAM.

199. The method of item 198, wherein the probe is labeled at the 3' end with a quencher selected from QSY, BHQ (Black Hole Quencher) and DFQ (Dark Fluorescent Quencher).

200. The method of item 193 or 194, wherein a positive control and a negative control are analyzed in conjunction with the sample.

201. The method of item 200, where in the positive control is a synthetic plasmid comprising targets from the coronavirus orf1ab gene, the S protein gene, the N protein gene and RNase P.

202. A composition for detecting the presence of SARS-CoV-2 in a DNA sample, comprising a nucleic acid primer containing a nucleic acid sequence selected from the group comprising any of the primers disclosed herein.

203. The composition of item 202, wherein the nucleic acid primer is a first forward primer configured to hybridize to one end of a first target sequence within a first target region in the SARS-CoV-2 viral RNA genome, or to a complement of the first target sequence.

204. The composition of item 202, wherein the nucleic acid sequence of the target region is SEQ ID NO: 1.

205. The composition of item 202, wherein the nucleic acid sequence of the target region is SEQ ID NO: 2.

206. The composition of item 202, wherein the nucleic acid sequence of the target region is SEQ ID NO: 3.

207. The composition of item 202-206, further including a first reverse primer configured to hybridize to the other end of the first target sequence or its complement.

208. The composition of any of the preceding items, further including a nucleic acid sample, a polymerase, a buffer and nucleotides.

209. The composition of any of items 202-208, further including a first probe containing a fluorescent or other detectable label.

210. The composition of item 209, wherein the label of the first probe is a fluorescent label and the oligonucleotide probe further includes a quencher that quenches the fluorescent label.

211. The composition of item 209 or 210, wherein the first probe is configured to hybridize to a first target subsequence that is complementary or identical to at least 10 contiguous nucleotides within the first target sequence, a DNA copy thereof, or to a complement of the first target sequence or its DNA copy.

212. A composition for amplifying one or more target sequences in the SARS-CoV-2 genome, comprising: a first forward primer and a first reverse primer configured to amplify a first target sequence present in a first target region of the SARS-CoV-2 genome, wherein the first target sequence includes at least 10 contiguous nucleotides of the first target region, the first target region having less than 50%, 40%, 30%, 20% or 10% identity with bat-SL-CoVZC45 coronavirus and with SARS-CoV-2.

213. The composition of item 212, wherein the first forward primer and the first reverse primer are configured to hybridize to different ends of the first target sequence, a DNA copy thereof, or their respective complements.

214. The composition of item 212 or 213, wherein the nucleic acid sequence of the first target region is SEQ ID NO: 1.

215. The composition of item 212 or 213, wherein the nucleic acid sequence of the first target region is SEQ ID NO: 2.

216. The composition of item 212 or 213, wherein the nucleic acid sequence of the first target region is SEQ ID NO: 3.

217. The composition of any of items 212-217, further including a nucleic acid sample, a polymerase, a buffer and nucleotides.

218. The composition of any of items 212 to 217, further including a first probe containing a fluorescent or other detectable label.

219. The composition of item 218, wherein the label of the first probe is a fluorescent label and the probe further includes a quencher that quenches the fluorescent label.

220. The composition of item 218 or 219, wherein the first probe is configured to hybridize to a first target subsequence that is complementary or identical to at least 10 contiguous nucleotides of the first target sequence, a DNA copy thereof, or their respective complements.

221. The composition of any of items 212-220, further including a second forward primer and a second reverse primer configured to amplify a second target sequence within a second target region of the SARS-CoV-2 genome.

222. The composition of item 221, wherein the second forward primer and the second reverse primer are configured to bind to different ends of the second target sequence or to a cDNA complement thereof.

223. The composition of items 221 or 222, wherein the nucleic acid sequence of the first target region and the second target region are different and are selected from the group consisting of: SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3.

224. The composition of items 221-223, further including a third forward primer and a third reverse primer configured to amplify a third target sequence within a third target region of the SARS-CoV-2 genome.

225. The composition of item 224, wherein the third forward primer and the third reverse primer are configured to bind to different ends of the third target sequence or to a DNA copy or DNA complement thereof.

226. The composition of items 224 and 225, wherein the nucleic acid sequence of the first target region, the second target region and the third target region are different and are selected from the group consisting of: SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3.

227. The composition of item 226, wherein the primer sequences specific for the first target sequence are selected from SEQ ID NOs: 248 and 487, or 211 and 501.

228. The composition of item 226 or 227, wherein the primer sequences specific for the second target sequence are selected from SEQ ID NOs: 5 and 441, or 100 and 337.

229. The composition of any of items 226-228, wherein the primer sequences specific for the third target sequence are selected from SEQ ID NOs: 4 and 320, or 34 and 423, or 160 and 468.

230. A composition useful as an amplification control in a reaction to detect SARS-CoV-2 viral nucleic acids in a sample, comprising a linear or circular nucleic acid molecule including, in any order, at least one target sequence derived from the N gene of SARS-CoV-2, at least one further target sequence derived from the S gene of SARS-CoV-2, and at least one target sequence derived from the orf1ab gene of SARS-CoV-2.

231. A kit for detecting SARS-CoV-2 viral nucleic acid in a sample, comprising a composition of any of items 202-230, or any combinations thereof.

232. The kit of item 231, further including a master mix.

233. The kit of item 232, wherein the master mix is TaqMan Fast Virus 1-Step Master Mix or TaqPath 1-Step RT-qPCR Master Mix, CG.

234. The kit of any of items 231-233, wherein at least one of the components is dried or freeze dried.

235. The kit of any of items 231-234, further including an array of qPCR assays, each qPCR assay situated in a different locus of the array.

236. The kit of item 235, wherein the different locus includes a well, channel, groove, cavity, site or feature on the surface of the array.

237. A method of detecting SARS-CoV-2 viral nucleic acid present in a sample, comprising:

(c) providing a composition according to any of items 202-230;

(d) forming a reaction volume by contacting the composition, in any order or combination, with a polymerase, nucleotides and a sample obtained from bodily tissue taken from an organism; and

(e) forming one or more amplification products containing amplified coronaviral sequences in the reaction volume, wherein the forming includes subjecting the reaction volume to amplification conditions suitable to amplify target coronaviral sequences from coronaviral nucleic acid, wherein the coronaviral nucleic acid are present in the sample prior to amplification.

238. The method of item 237, further including detecting at least one of the amplification products during or after the forming step.

239. The method of item 238, further including diagnosing a coronaviral infection in the organism.

240. The method of items 237-239, wherein the organism is a human patient and the coronaviral nucleic acid is derived from SARS-CoV-2.

241. The method of items 237-240, wherein the forming includes amplifying at least three different coronaviral target sequences from the coronaviral nucleic acid.

242. The method of item 241, wherein the at least three different coronaviral target sequences include one target sequence derived from the N gene, one target sequence derived from the S gene, and one target sequence derived from the orf1ab gene.

APPENDIX 1

applied biosystems PRODUCT INFORMATION SHEET

TaqMan™ 2019 nCoV Assay Kit v1

For use with TaqMan™ 2019 nCoV Control Kit v1 for detection of the 2019 novel coronavirus

Catalog Number A47532

Pub. No. MAN0019096 **Rev.** A.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product descriptions

Applied Biosystems™ TaqMan™ 2019 nCoV Assay Kit v1 (Cat. No. A47532) contains a set of TaqMan™ RT-PCR assays for the *in vitro* qualitative detection and characterization of the 2019 novel coronavirus (nCoV) in respiratory samples. The kit includes three assays that target nCoV genes, and one positive control assay that targets the Human RNase P gene:

- nCoV Assays target three different virus genomic regions, reducing the risk of false negatives.
- nCoV Assays have undergone bioinformatics selection and analysis to specifically target sequences that are unique to 2019 nCoV.
- The RNase P Assay serves as an internal positive control for each reaction, enabling relative quantification of viral genes.
- The nCoV Assays perform well with total nucleic acid that is isolated from nasopharyngeal swab, nasopharyngeal aspirate, and bronchoalveolar lavage (BAL) samples.

The TaqMan™ 2019 nCoV Assay Kit v1 is used with the TaqMan™ 2019 nCoV Control Kit v1 (Cat. No. A47533) to monitor assay-specific amplification.

Contents and storage

Contents	Dye	Amount[1]	Concentration	Storage
nCoV (Orf-1ab) (Tube 1)	FAM™ dye	75 µL	20X	−30°C to −10°C
nCoV (S Protein) (Tube 2)	FAM™ dye	75 µL	20X	
nCoV (N Protein) (Tube 3)	FAM™ dye	75 µL	20X	
RNase P Assay (Tube 4)	VIC™ dye	250 µL	20X	

[1] Sufficient for 50 × 25-µL reactions.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Item	Source
Real-Time PCR Instrument (one of the following)	
7500 Real-Time PCR Instrument	Contact your local sales office
QuantStudio™ 5 Real-Time PCR System (96-well, 0.2-mL Block)	
QuantStudio™ 3 Real-Time PCR System (96-well, 0.2-mL Block)	
Equipment	
Vortex mixer	MLS
Micropipettes	
Microcentrifuge	

Item	Source
Reagents	
TaqMan™ 2019 nCoV Control Kit v1	A47533
Master Mix (one of the following)[1]: • TaqPath™ 1-Step RT-qPCR Master Mix, CG • TaqMan™ Fast Virus 1-Step Master Mix (contains dUTP/UNG)	 • A15299, A15300 • 4444432, 4444434, 4444436
RT-PCR Grade Water	AM9935
Tubes, plates, and other consumables	
MicroAmp™ Optical 96-Well Reaction Plate (0.2-mL)	N8010560, or equivalent[2]
MicroAmp™ Optical Adhesive Film	4306311
Aerosol-resistant barrier pipette tips	MLS
Disposable gloves	MLS
Software	
(*Recommended*) QuantStudio™ Design and Analysis Software v2	thermofisher.com/qpcrsoftware

[1] Other Master Mixes are supported, but have not been tested.
[2] See thermofisher.com/plastics.

Procedural guidelines

Guidelines for nucleic acid isolation

- The assays perform well with total nucleic acid that is isolated from nasopharyngeal swab, nasopharyngeal aspirate, and bronchoalveolar lavage (BAL) samples.
- Use your preferred method of nucleic acid isolation.
- To perform nucleic acid isolation from respiratory tract samples using the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit, see "Related documentation" on page 2.

Guidelines for 1-Step RT-PCR

- Use purified, nondegraded total nucleic acid that is dissolved in PCR-compatible buffer.
- Ensure that the input nucleic acid is free of RNase activity and inhibitors of reverse transcription (RT) and PCR.
- Protect the assays from light and store as indicated until ready for use. Excessive light exposure can negatively affect the fluorescent probes.
- To avoid false negatives, test each sample with all three of the nCov Assays.
- Include the RNase P Assay as an internal positive control.
- Before you begin, determine the number of reactions that are required. Include the following reactions for each assay:

Table 1 Determine the number of reactions for each nCoV Assay

Reaction type	Number of reactions
Nucleic acid isolated from respiratory sample	Number of samples × Number of replicates[1]
nCoV Control v1	1 per nCoV Assay per plate
No-template control (NTC) or negative extraction control (NEC)	1 per nCoV Assay per plate

[1] (*Optional*) Run technical replicates in triplicate to identify outliers.

Perform 1-Step RT PCR

Prepare 1-Step RT-PCR reactions

IMPORTANT! For optimal results, prepare the reactions on ice.

Thaw the master mix and purified nucleic acid samples on ice.

DOCKET NO. LT04529PCT

1. Resuspend the samples by inverting the tube, then gently vortexing.
2. Gently swirl the master mix tube to mix thoroughly. Do not invert the tube, as this can introduce bubbles.
3. For each nCoV Assay, combine the following components for the number of required reactions, plus 10% overage:

Component	Volume for 1 reaction
Master Mix (4X)	6.25 µL
nCoV Assay (20X)	1.25 µL
RNase P Assay (20X)	1.25 µL
RT-PCR Grade Water	11.25 µL
Total RT-PCR Reaction Mix volume	**20.0 µL**

4. Vortex the RT-PCR Reaction Mix tubes (1 for each nCoV Assay), then centrifuge briefly.
5. For each nCoV Assay, combine the following components in the appropriate wells of a MicroAmp™ Optical 96-Well Reaction Plate (0.2-mL).

Component	Volume per well
RT-PCR Reaction Mix (see step 3)	20.0 µL
One of the following: • Nucleic acid sample *or* • nCoV Control v1 + 4 µL Nuclease-free Water *or* • Negative control (NTC or NEC)	5.0 µL
Total volume	**25.0 µL**

6. Seal the plate with a MicroAmp™ Optical Adhesive Film, then vortex briefly to mix the contents.
7. Centrifuge the plate briefly to collect the contents at the bottom of the wells.

IMPORTANT! Run the plate within 2 hours of preparation, or store the plate in the dark at 2–8°C for up to 24 hours.

Set up and run the real-time PCR instrument

See the appropriate instrument documentation for detailed instructions to program the thermal-cycling conditions or to run the plate.

1. Select the cycling mode: **Standard**.
2. Set the ramp rate:
   - QuantStudio™ 3 or 5 System: 1.6°C/second
   - 7500 Real-Time PCR System: 100%
3. Set up the thermal protocol.

Stage	Step	Temperature	Time
Hold	UNG incubation[1]	25°C	2 minutes
Hold	Reverse transcription	50°C	• TaqPath™ 1-Step RT-qPCR Master Mix, CG: 15 minutes • TaqMan™ Fast Virus 1-Step Master Mix: 5 minutes
Hold	Activation[2]	95°C	• TaqPath™ 1-Step RT-qPCR Master Mix, CG: 2 minutes • TaqMan™ Fast Virus 1-Step Master Mix: 20 seconds
Cycling (40 cycles)	Denaturation	95°C	3 seconds
	Anneal/Extension	60°C	30 seconds

[1] Heat-labile UNG is completely inactivated during the first ramp to 95°C.
[2] Required for RT inactivation, first denaturation, and activation of the DNA polymerase.

4. Set the reaction volume: 25 µL.

5. Load the plate into the real-time PCR instrument, then start the run.

Guidelines for data analysis

For detailed information about data analysis, refer to the software Help or user guide.

1. (*Recommended*) Use QuantStudio™ Design and Analysis Software v2 for data analysis.
2. Analyze data using the Baseline Threshold Algorithm, Automatic Threshold, and Automatic Baseline analysis settings.
3. Review results for each reaction in the amplification plot (in log or linear view).
   Assess the overall shape of the curve, then adjust baseline and threshold values, if needed.
4. Confirm that the control reactions for each nCoV Assay perform as expected:

   Table 2 Expected results of the control reactions for each nCoV Assay

Reaction	Expected C_t value	Expected Amp Status	About unexpected results
NTC or NEC	Undetermined	No Amp	If the NTC or NEC have an amplification curve that crosses the threshold (false positive), sample contamination may have occurred. Repeat the test with new reagents, following good RT-PCR practices.
nCoV Control v1	Ct ⩽ 37 Amp	Amp	If expected positive reactivity is not achieved, repeat the test with new reagents.

5. Classify the results for the individual nCoV Assays, according to the C_t value and amplification status, as indicated in the table:

   Table 3 Individual assay result interpretation

	C_t value	Amp status	nCoV Assay Result
nCoV Assay	C_t ⩽ 37	Amp	Positive[1]
		Inconclusive	Inconclusive. Repeat the test.
	37 < C_t < 40	Amp *or* Inconclusive	Inconclusive. Repeat the test using >5 µL of the sample.
	C_t = Undetermined	No Amp	Negative
RNAseP Assay	C_t ⩽ 37	Amp	Positive
	C_t = Undetermined	No Amp	Negative

[1] A C_t value of 35±2 indicates the presence of10 copies of the viral gene. This corresponds to 2 copies/µL in the extracted nucleic acid sample.

6. Review all of the assays for a single sample, then interpret the results using the table:

   Table 4 Combined Assay results interpretation

nCoV Assay results	RNAse P Assay results	Interpretation of results
All three positive	Positive or Negative	nCoV is present in the sample.
All three negative	Positive	nCoV is not present in the sample.
	Negative	Invalid result.
One or two positive	Positive or Negative	Interpret results according to guidelines established in your lab.

DOCKET NO. LT04529PCT

Related documentation

Document	Publication Number
TaqMan™ 2019 nCoV Control Kit v1 Product Information Sheet	MAN0019097
TaqMan™ Gene Expression Assays User Guide—single-tube assays	4333458
Isolation of Nucleic Acid for Respiratory Tract Microbiota Profiling Experiments Quick Reference	MAN00018526
TaqMan™ Multiplex PCR Optimization User Guide	MAN0010189

Limited product warranty

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

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Revision history: Pub. No. MAN0019096

Revision	Date	Description
A.0	6 February 2020	New document for release of TaqMan™ 2019 nCoV Assay Kit v1.

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APPENDIX 2

Appendix 2

TaqMan™ 2019-nCoV Multiplex Assay Kit v1

For use with TaqMan™ 2019-nCoV Multiplex Control Kit v1 for detection of the 2019 novel coronavirus RNA (2019-nCoV)

TaqMan® Assays for detection on 96-well 0.2 ml plates

for use with:

7500 Real-Time PCR System

QuantStudio™ 5 Flex Real-Time PCR Systems

Product description

Applied Biosystems™ TaqMan™ 2019-nCoV Multiplex Assay Kit v1 (Cat. No. ANNNNN) contains a set of TaqMan™ RT-PCR assays for the qualitative detection and characterization of the 2019 novel coronavirus (2019-nCoV) RNA.

The kit includes three assays that target viral genes, and one positive control assay that targets an exogenous control RNA from bacteriophage M2:

- Assays target three different virus genomic regions, (ORF1ab, N and S genes) reducing the risk of false negatives.
- Assays have undergone bioinformatics selection and analysis to specifically target sequences that are unique to 2019-nCoV.
- The MS2 Assay is run in multiplex with each 2019-nCoV Assay as an exogenous internal positive control.
- The 2019-nCoV Assays perform well with total nucleic acid that is isolated from research samples collected via nasopharyngeal swab, nasopharyngeal aspirate, and bronchoalveolar lavage (BAL).

The kit is used with the TaqMan™ 2019-nCoV Multiplex Control Kit v1 (Cat. No. ANNNNN) to monitor assay-specific amplification.

For more information about genetic analysis solutions available for 2019-nCoV analysis, go to thermofisher.com/coronavirus.

Contents and storage

nCoV Multiplex Component	Dye Label/ Quencher	Targets	Accession Number	Amount [1]	Concentration	Storage
Polyprotein ORF 1ab Assay	6FAM/QSY	ORF 1ab	MN908947.3	75 µl	20X	-25°C to -15°C
S Protein Assay	ABY/QSY	S Protein				
N Protein Assay	VIC/QSY	N Protein				
Internal Positive Control Assay	JUN/QSY	Exogenous MS2 RNA template	NA			
Internal Positive Control RNA Template				50 µl	25X	-25°C to -15°C

[1] Sufficient for 50 × 25-µL reactions.

Some materials may not be supplied

Item	Source
Real-time PCR instrument (one of the following)	
QuantStudio™ 5 Real-Time PCR System (96-well, 0.2-mL Block)	Contact your local sales office
7500 Real-Time PCR System	
[redacted]	
TaqMan™ 2019-nCoV Multiplex Control Kit v1	ANNNNN
TaqPath™ 1-Step Multiplex Master Mix (NO ROX)	A28521, A28522, A28523

Software

Item	Documentation
QuantStudio™ Design and Analysis Software v2.3	QuantStudio™ Design and Analysis Software v2 USER GUIDE, Publication Number MAN0018200

Procedural guidelines

• Use purified, non-degraded total nucleic acid that free of RNase activity and RT-PCR inhibitors.

Note: To perform nucleic acid isolation using the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit, see "Related documentation."

• Protect the assays from light.

• To avoid contamination, do not use the same pipette for positive controls and samples to avoid contamination.

• For each reaction, run the 2019-nCov Assay as a 4-plex with the MS2 Assay.

• Before you begin, determine the number of required reactions. Include the following reactions for each assay:

Sample type	Number of reactions
Nucleic acid test sample	Number of samples × Number of replicates [1]
2019-nCoV Multiplex Control v1	2 per plate
No-template control (NTC) or negative extraction control (NEC)	2 per plate

[1] Replicates are optional.

Perform 1-Step RT-PCR

IMPORTANT! For optimal results prepare the plate on ice.
Use a separate pipette for the control template to avoid contamination.
Run the plate within 2 hours of preparation or store the plate at 2–8°C for up to 24 hours.

1. Thaw the purified nucleic acid samples, master mixes, and positive control template on ice. Resuspend the samples by gently vortexing and spinning sample tubes in a microcentrifuge.

2. Mix the 1-Step Master Mix thoroughly but gently to avoid bubbles.

3. For each test, combine the following components for the number of reactions, plus 10% overage:

Component	Volume per sample	Volume per positive control	Volume per NTC or negative extraction control
TaqPath 1-Step Multiplex Master Mix (4X)	6.25 µL		
nCoV Multiplex Assay (20X)	1.25 µL		
Nuclease-free water	12.50 µL		
Internal Positive Control Template	1.00 µL		
Total PCR Reaction Mix volume	**20.00 µL**		

Pipette 20 ul of PCR reaction mix to each well of a MicroAmp™ Optical 96-Well Reaction Plate (0.2-mL) well and combine with sample or control as shown below.

Component	Volume per reaction
Reaction Mix (see step 1)	20.0 µL
· Nucleic acid sample or · Positive control mix containing 1 µL 2019-nCoV Multiplex Control v1 [1] and 4 µL RT-PCR Grade Water or · Negative control (nuclease-free water for NTC or NEC)	5.00 µL
Total reaction volume	**25.00 µL**

[1] Use a separate pipette for the control template to avoid sample contamination.

4. Using the instrument software, load and run the reactions on a real-time PCR instrument using a Standard ramp (1.6 °C /sec) and the following thermal protocol:

Stage	Step	Temperature	Time
Hold	UNG incubation [1]	25°C	2 minutes
Hold	Reverse transcription	53°C	10 minutes
Hold	Activation [2]	95°C	2 minutes
Cycling (40 Cycles)	Denaturation	95°C	3 seconds
	Anneal/Extension	60°C	30 seconds

[1] Heat-labile UNG is completely inactivated during the first ramp to 95°C.

[2] Used for RT inactivation, first denaturation, and activation of the DNA polymerase.

Guidelines for data analysis

For detailed information about data analysis, refer to the Software Help or User Guide.

(Recommended) Use QuantStudio™ Design and Analysis Software v2 for data analysis.

1. Open the eds file in the QuantStudio Design & Analysis v2.3 software.
2. Analyze data for all assays using the default Automatic Threshold and Automatic Baseline analysis settings.
3. Review results for each positive and negative control reaction to make sure that amplification is visible in all positive controls for all assays, and that there is no amplification in the NTC or NEC reactions. For positive controls if Ct > 37 for any assay, this indicates qPCR reaction failure. Amplification in negative samples with Ct ≤ 37 suggests possible reagent or pipette contamination. Repeat the test with new materials if either type of control assay fails to meet these criteria

Table 1 Expected results of the control reactions for each 2019-nCoV Assay

Reaction	Expected Ct value	About unexpected results
NTC or NEC	Undetermined	If the NTC or NEC has an amplification curve that crosses the threshold (false positive), sample contamination may have occurred. Repeat the test with new reagents, following good RT-PCR practices.
2019-nCoV Control v1	Ct ≤ 37 Amp	If expected positive reactivity is not achieved, repeat the test with new reagents
MS2 IPC Assay	Ct ≤ 37	qPCR reagents are functional
	Ct = Undetermined	qPCR reaction failure

4. Omit the positive wells from the plate and analyze the data again to maximize sensitivity of detection.
5. Under Actions click Export. In the next window check the box to combine all reports in one file.
6. Browse for the directory and export the data.
7. Classify the results for the individual assays using the following table.

Results interpretation

1. FAM channel detects SARS-CoV-2 viral target gene ORF1ab; VIC channel detects SARS-CoV-2 viral target gene N gene, and ABY channel detects SARS-CoV-2 viral target gene S gene. JUN channel detects internal positive control MS2.

2. Interpretation of viral target genes:

Target	Channel	Scenario 1	Scenario 2	Scenario 3
ORF1ab	FAM	Ct value<37	37≤Ct value<40	No Ct value or Ct value =40
	Results judged	Positive*	Indeterminate**	Negative***
N gene	VIC	Ct value<37	37≤Ct value<40	No Ct value or Ct value =40
	Results judged	Positive*	Indeterminate**	Negative***
S gene	ABY	Ct value<37	37≤Ct value<40	No Ct value or Ct value =40
	Results judged	Positive*	Indeterminate**	Negative***

* Scenario 1 (Positive): the Ct value is < 37, the amplification curve is S-shaped.

** Scenario 2 (Indeterminate): the amplification curve is S-shaped, and 37 ≤Ct <40, which needs to be re-run; if the re-run results Ct < 37 or are consistent with first run 37 ≤Ct < 40 with S-shaped amplification curve, the result is determined to be positive for the specific gene target.

*** Scenario 3 (Negative): If MS2 (JUN channel) has no Ct value or Ct value=40, run of multiplex is invalid.

Note: When viral target concentration is ≥ 10^5 copies/Rxn, MS2 Ct value will be higher than that of the positive control.

3. SARS-CoV-2 nucleic acid test positive interpretation criteria:

(1) Any two of the three genes tested are positive [ORF1ab and N genes (and or S genes); ORF1ab and S genes (and or N genes); N and S genes (and or ORF1ab genes)].

(2) Any one of the three genes (ORF1ab or N or S genes) is positive in two different sample collections of the same subject.

The above set of criteria are recommendations based on analytical analysis. Please determine the criteria for your use case based on your accuracy testing in your lab with appropriate samples.

APPENDIX 3

appliedbiosystems

Respiratory Tract Microbiota Profiling Experiments

APPLICATION GUIDE

TaqMan® Assays for respiratory tract microbiota profiling experiments in TaqMan® Array Card format

for use with:
TaqMan® Array Respiratory Tract Microbiota Comprehensive Card
Custom TaqMan® Array Cards
MagMAX™ Viral/Pathogen Ultra Nucleic Acid Isolation Kit
QuantStudio™ 12K Flex Real-Time PCR System
QuantStudio™ 7 Flex Real-Time PCR System

Catalog Number A41238
Publication Number MAN0017951
Revision C.0

For Research Use Only. Not for use in diagnostic procedures.

ThermoFisher SCIENTIFIC

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

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Revision history: Pub. No. MAN0017951

Revision	Date	Description
C.0	09 September 2019	• Updated product information for TaqMan® Array Respiratory Tract Microbiota Comprehensive Card and TaqMan® Universal Extraction Control Organism (*B. atrophaeus*) • Removed Early Access designation
B.0	25 July 2019	• Updated data analysis recommendations • Updated nucleic acid isolation kit product information and workflow • Updated control product information • Changed master mix for preamplification to TaqPath™ 1-Step RT-qPCR Master Mix, CG • Changed master mix for array card real-time PCR to TaqMan® Fast Advanced Master Mix, No UNG
A.0	6 August 2018	New document.

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1 Overview

Product information

TaqMan® Array Respiratory Tract Microbiota Comprehensive Card (Cat. No. A41238) is an efficient, easy-to-use TaqMan® Array Card for the characterization of key respiratory tract microbes. The array card includes TaqMan® assays that have been optimized for detection of 42 respiratory tract viral and bacterial, and fungal microbes. The array card also includes control assays for TaqMan® Universal Extraction Control Organism (*B. atrophaeus*), TaqMan® Universal RNA Spike In/Reverse Transcription (Xeno) Control, the human RNase P RPPH1 gene, and the human 18S ribosomal RNA gene.

The assays perform well with total nucleic acid that is isolated from nasopharyngeal swab, nasopharyngeal aspirate, and bronchoalveolar lavage (BAL) samples using the MagMAX™ Viral/Pathogen Ultra Nucleic Acid Isolation Kit (see Chapter 2, "Isolate nucleic acid using MagMAX™ Viral/Pathogen Ultra Nucleic Acid Isolation Kit").

TaqMan® assay designs and assay target sequences have undergone rigorous bioinformatics selection and analysis to maximize strain coverage and minimize potential for off-target cross-reactivity. Qualified TaqMan® assays also undergo performance testing to ensure that results are accurate and reproducible with high levels sensitivity and specificity.

TaqMan® assays for respiratory tract microbiota profiling

The following assays are included in the TaqMan® Array Respiratory Tract Microbiota Comprehensive Card.

The assays can also be ordered in a Custom TaqMan® Array Card.

Table 1 Assays for respiratory tract microbiota targets

Target organism	Assay name	Nucleic acid type	Assay ID
Bacteria			
Bordetella bronchiseptica / parapertussis / pertussis	Bordetella	DNA	Ba06439624_s1
Bordetella holmesii	B.holmesii	DNA	Ba06439621_s1
Bordetella pertussis	B.pertussis	DNA	Ba06439623_s1
Chlamydophila pneumoniae	C.pneumoniae	DNA	Ba06439616_s1
Coxiella burnetii	C.burnetii	DNA	Ba06439618_s1
Haemophilus influenzae	H.influenzae	DNA	Ba06439625_s1

Target organism	Assay name	Nucleic acid type	Assay ID
Klebsiella pneumoniae	K.pneumoniae	DNA	Ba04932083_s1
Legionella pneumophila	L.pneumophila	DNA	Ba06439617_s1
Moraxella catarrhalis	M.catarrhalis	DNA	Ba06439622_s1
Mycoplasma pneumoniae	M.pneumoniae	DNA	Ba06439620_s1
Staphylococcus aureus	S.aureus	DNA	Ba04646259_s1
Streptococcus pneumoniae	S.pneumoniae	DNA	Ba06439619_s1
Fungus			
Pneumocystis jirovecii	P.jirovecii	DNA	Fn06439626_s1
Virus			
Adenovirus	AdV_1of2	DNA	Vi99990001_po
Adenovirus	AdV_2of2	DNA	Vi99990002_po
Human Bocavirus	HBoV	DNA	Vi99990003_po
Human Coronavirus 229E	CoV_229E	RNA	Vi06439671_s1
Human Coronavirus HKU1	CoV_HKU1	RNA	Vi06439674_s1
Human Coronavirus NL63	CoV_NL63	RNA	Vi06439673_s1
Human Coronavirus OC43	CoV_OC43	RNA	Vi06439646_s1
Human Enterovirus (pan assay)	EV_pan	RNA	Vi06439631_s1
Human Enterovirus D68	EV_D68	RNA	Vi06439669_s1
Human Metapneumovirus (hMPV)	hMPV	RNA	Vi99990004_po
Human Parainfluenza virus 1	hPIV1	RNA	Vi06439642_s1
Human Parainfluenza virus 2	hPIV2	RNA	Vi06439672_s1
Human Parainfluenza virus 3	hPIV3	RNA	Vi06439670_s1
Human Parainfluenza virus 4	hPIV4	RNA	Vi99990005_po
Human Parechovirus	HPeV	RNA	Vi99990006_po
Human Respiratory Syncytial Virus A (RSVA)	RSVA	RNA	Vi99990014_po
Human Respiratory Syncytial Virus B (RSVB)	RSVB	RNA	Vi99990015_po
Human Rhinovirus 1/2	RV_1of2	RNA	Vi99990016_po
Human Rhinovirus 2/2	RV_2of2	RNA	Vi99990017_po
Human herpesvirus 3 (HHV3 – Varicella zoster Virus)	HHV3	DNA	Vi06439647_s1

Target organism	Assay name	Nucleic acid type	Assay ID
Human herpesvirus 4 (HHV4 – Epstein-Barr Virus)	HHV4	DNA	Vi06439675_s1
Human herpesvirus 5 (HHV5 – Cytomegalovirus)	HHV5	DNA	Vi06439643_s1
Human herpesvirus 6 (HHV6)	HHV6	DNA	Vi06439627_s1
Influenza A	Flu_A_pan	RNA	Vi99990011_po
Influenza A/H1-2009	Flu_A_H1	RNA	Vi99990009_po
Influenza A/H3	Flu_A_H3	RNA	Vi99990010_po
Influenza B	Flu_B_pan	RNA	Vi99990012_po
Measles virus	Measles	RNA	Vi99990013_po
Middle East Respiratory Syndrome coronavirus (MERS)	MERS_CoV	RNA	Vi06439644_s1
Mumps virus	Mumps	RNA	Vi06439657_s1
Severe Acute Respiratory Syndrome coronavirus (SARS)	SARS_CoV	RNA	Vi06439634_s1

Table 2 Assays for respiratory tract microbiota controls

Control name	Assay name	Nucleic acid type	Assay ID
TaqMan® Universal Extraction Control Organism (*B. atrophaeus*)	B.atrophaeus	DNA	Ba06596576_s1
TaqMan® Universal RNA Spike In/Reverse Transcription (Xeno) Control	Xeno	RNA	Ac00010014_a1
Human RNase P RPPH1 gene	RPPH1	DNA	Hs04930436_g1
Human 18S ribosomal RNA gene[1]	18S	DNA	Hs99999901_s1

[1] Human 18S ribosomal RNA is used as a manufacturing control for the array card and is not an assay specific to Respiratory Tract Microbiota profiling.

TaqMan® Array Card products and formats

TaqMan® Array Respiratory Tract Microbiota Comprehensive Card

The TaqMan® Array Respiratory Tract Microbiota Comprehensive Card (Cat. No. A41238) contains pre-plated, dried down TaqMan® assays for respiratory tract microbiota profiling. For the complete lists of assays included with the array card, see "TaqMan® assays for respiratory tract microbiota profiling" on page 5.

Contents and storage

Table 3 TaqMan® Array Respiratory Tract Microbiota Comprehensive Card (Cat. No. A41238)

Component	Amount	Storage
TaqMan® Array Respiratory Tract Microbiota Comprehensive Card	10 cards[1]	2°C to 8°C

[1] Minimum order.

Custom TaqMan® Array Card formats

Any of the TaqMan® assays for respiratory tract microbiota profiling can be included in a Custom TaqMan® Array Card.

Array format	Manufacturing controls	Number of assays/replicated	Maximum number of samples
24	1	23/2	8
48	1	47/1	8

Configure and order Custom TaqMan® Array Cards

1. Go to **thermofisher.com/arraycards**.
2. In the table, click **Select** to configure a 24 or 48 format array card.
   The **Custom Array Configurator** screen displays.
3. Enter the custom array name in the **Array name** text field.
4. Click **Import Your Assay List**, then upload or copy-paste the assay information:
   - Under **Upload a list of Assay IDs**, click **Choose File**, then select a tab-delimited text file (TXT) containing Assay IDs.
     or
   - Under **Enter a list of Assay IDs**, paste the Assay IDs, then click **Import Entered List**.
5. Follow the on-screen instructions to configure the assays on the plate.
6. *(Optional)* Click **Save Your Array** at any time to save the array configuration to your Thermo Fisher Scientific account.
7. When the plate is configured, click **Complete Your Design**, then follow the on-screen instructions to complete the order.

Materials required but not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Materials required for nucleic acid isolation

Nucleic acid isolation kit

Table 4 MagMAX™ Viral/Pathogen Ultra Nucleic Acid Isolation Kit (Cat. No. A42356)

Component	Amount	Storage
Binding Solution	60 mL	15°C to 25°C
Wash Buffer	100 mL	
Elution Solution	10 mL	
Nucleic Acid Binding Beads	2 mL	
Proteinase K	1 mL	
Enzyme Mix	5 mL	–25°C to –15°C

Additional materials

Item	Source
Instrument and equipment	
KingFisher™ Flex Magnetic Particle Processor 96DW with deep-well heat block	5400630
Adjustable micropipettors	MLS
Multi-channel micropipettors	MLS
Plastics and consumables	
KingFisher™ Deepwell 96 Plate	95040450
KingFisher™ 96 KF microplate (200 µL)	97002540
KingFisher™ 96 tip comb for DW magnets	97002534
Conical Tubes (15 mL)	AM12500
Conical Tubes (50 mL)	AM12501
Nonstick, RNase-Free Microfuge Tubes, 1.5 mL	AM12450
Nonstick, RNase-Free Microfuge Tubes, 2.0 mL	AM12475
MicroAmp™ Clear Adhesive Film	4306311
Filtered micropipettor tips	MLS
Reagent reservoirs	MLS

Item	Source
Reagents	
Ethanol, 100% (molecular biology grade)	MLS
Nuclease-free Water	AM9932, or equivalent
Universal Transport Media, for preparation of negative extraction control	Fisher Scientific 22-031-14, or equivalent
(*Optional*) 1X PBS (1X), pH 7.4, for reconstitution of TaqMan® Universal Extraction Control Organism (*B. atrophaeus*)	10010023

Materials required for PCR

Table 5 Materials required for preamplification and real-time PCR

Item	Source
Equipment	
Microcentrifuge	MLS
Vortex mixer	MLS
Micropipettes	MLS
Tubes, plates, and other consumables	
MicroAmp™ Optical 96-Well Reaction Plate	N8010560, or equivalent; see thermofisher.com/plastics
MicroAmp™ Clear Adhesive Film	4306311
Aerosol-resistant barrier pipette tips	MLS
Disposable gloves	MLS
Reagents	
Nuclease-free Water	AM9937, or equivalent

Table 6 Additional materials required for preamplification

Item	Source
Thermal cycler, one of the following (or equivalent): • Veriti™ Thermal Cycler, 96-well standard block • SimpliAmp™ Thermal Cycler • ProFlex™ PCR System	Contact your local sales office
TaqPath™ 1-Step RT-qPCR Master Mix, CG	A15299
TaqMan® PreAmp Pool, Respiratory Tract Microbiota, 4X	4485255[1]

[1] To order, contact CustomControls@thermofisher.com.

Table 7 Additional materials required for real-time PCR with TaqMan® Array Cards

Item	Source
Real-time PCR instrument, one of the following; configured with the TaqMan® Array Card block and heated cover.	
QuantStudio™ 7 Flex Real-Time PCR System	Contact your local sales office
QuantStudio™ 12K Flex Real-Time PCR System	
Equipment	
TaqMan® Array Card Sealer	4331770
Centrifuge with custom buckets and card holders, one of the following: • Sorvall™ centrifuge • Heraeus™ centrifuge See the **Resources** section at **thermofisher.com/taqmanarrays** for a list of compatible centrifuges, rotors, and buckets.	Contact your local sales office
Blank balance TaqMan® Array Cards (Included with the instrument block upgrade / installation kit)	Contact your local sales office
Reagents	
TaqMan® Fast Advanced Master Mix, No UNG	• A44359 (1 mL) • A44360 (5 mL)

Materials required for data analysis

Item	Source
Software, select one of the following:	
Relative Quantification App (recommended)	apps.thermofisher.com
QuantStudio™ 12K Flex Software	Included with QuantStudio™ 12K Flex Real-Time PCR System
QuantStudio™ 6 and 7 Flex Software	Included with QuantStudio™ 6 and 7 Flex Real-Time PCR System

Optional controls

Control	Purpose	How to use	Cat. No.
TaqMan® Universal RNA Spike In/Reverse Transcription (Xeno) Control	Control for RNA recovery and purification, and reverse transcription	Nucleic acid isolation: Add to samples along with the Binding/Bead Mix	A39179
TaqMan® Universal Extraction Control Organism (*B. atrophaeus*)	Control for nucleic acid extraction and purification	Nucleic acid isolation: Stand-alone sample, or add to samples after Enzyme Mix	A39180
TaqMan® Respiratory Tract Microbiota Amplification Control	Control for real-time PCR	Real-time PCR: Stand-alone sample added directly to the card	A39178

TaqMan® Universal RNA Spike In/Reverse Transcription (Xeno) Control

TaqMan® Universal RNA Spike In/Reverse Transcription (Xeno) Control serves as an exogenous process control for nucleic acid isolation and RNA recovery, reverse transcription, and preamplification. The control can also be used as an internal positive control for real-time PCR. The control is used with the proprietary TaqMan® assay for Xeno™ sequences, which is included in the TaqMan® Array Respiratory Tract Microbiota Comprehensive Card.

TaqMan® Universal RNA Spike In/Reverse Transcription (Xeno) Control is supplied at a concentration of 10,000 copies/µL. During nucleic acid isolation, 10 µL of the control can be added to each test sample along with the binding reagents. When carried through the respiratory tract microbiota workflow, the control is used to monitor nucleic acid recovery and to indicate the presence of PCR inhibitors. Using a control to monitor sample-specific PCR inhibition reduces the likelihood of false negatives and provides confidence that results are accurate.

TaqMan® Universal Extraction Control Organism (*B. atrophaeus*)

TaqMan® Universal Extraction Control Organism (*B. atrophaeus*), serves as a process control for cell lysis and nucleic acid recovery. The control is used with the proprietary TaqMan® assay for *Bacillus atrophaeus* sequences.

Like other gram-positive bacteria, *Bacillus atrophaeus* has thick cell walls than can be difficult to lyse. This characteristic makes gram-positive bacteria an ideal control to monitor the efficiency of cell lysis and subsequent nucleic acid recovery.

TaqMan® Universal Extraction Control Organism (*B. atrophaeus*) is supplied lyophilized with a quantity of 1×10^9 copies/vial, and is reconstituted in 200 µL of 1X PBS (1X), pH 7.4 to a final concentration 5×10^6 copies/µL. During nucleic acid isolation, 10 µL of the control is processed as a stand-alone sample in a background of universal transport media. It can also be added to one or more test samples at the start of the extraction process. The control is carried through the remainder of the workflow with the test samples. It is recommended that at least one stand-alone control sample is run per extraction plate.

TaqMan® Respiratory Tract Microbiota Amplification Control

TaqMan® Respiratory Tract Microbiota Amplification Control contains a linearized multi-target plasmid with target sequences for each available respiratory tract microbiota profiling assay. The plasmid also contains target sequences for TaqMan® Universal RNA Spike In/Reverse Transcription (Xeno) Control and the human RNase P RPPH1 gene. It can be included in respiratory tract microbiota profiling experiments as a positive control for panel-specific amplification.

TaqMan® Respiratory Tract Microbiota Amplification Control is supplied at a concentration of 1×10^5 copies/µL. During real-time PCR, 10 µL of the control is used as a stand-alone sample in one port of the TaqMan® Array Respiratory Tract Microbiota Comprehensive Card. The control can be used if needed to verify assay performance and to help with troubleshooting.

Note: The amplification control RV target sequence is a perfect match to the RV_1of2 assay target, and contains a mismatch with the RV_2of2 assay target. Lower Amp Scores and C_q confidence scores can be noted for RV_2of2 versus RV_1of2.

Workflow using TaqMan® Array Cards

Start with bronchoalveolar lavage, nasopharyngeal swab, or nasopharyngeal aspirate samples

▼

Isolate nucleic acid using MagMAX™ Viral/Pathogen Ultra Nucleic Acid Isolation Kit (page 15)

▼

Perform preamplification (page 20)

▼

Prepare and run TaqMan® Array Cards (page 23)

▼

Analyze data (page 25)

2 Isolate nucleic acid using MagMAX™ Viral/Pathogen Ultra Nucleic Acid Isolation Kit

For required materials, see "Materials required for nucleic acid isolation" on page 10.

Procedural guidelines

- Perform all steps at room temperature (20–25°C) unless otherwise noted.
- Ensure that Nucleic Acid Binding Beads remain in a homogeneous suspension while pipetting. Vortex beads before use.

Before first use of the kit

- Download the KingFisher™ Flex script **MVP_Ultra_Flex** from the product page, then install it on the instrument.

  See the instrument user guide for instructions to install the script.

- Prepare fresh 80% Ethanol using 100% absolute Ethanol and Nuclease-free Water, sufficient for 1.5 mL per sample, plus 10% overage.

Set up the KingFisher™ Flex instrument

- Ensure that the KingFisher™ Flex instrument has the appropriate magnetic head and heat block installed.
  - 96 deep-well magnetic head
  - 96 deep-well heat block
- Ensure that the **MVP_Ultra_Flex** script is installed on the instrument.

Reconstitute TaqMan® Universal Extraction Control Organism (*B. atrophaeus*)

Use of the TaqMan® Universal Extraction Control Organism (*B. atrophaeus*) is optional.

1. Remove metal fastener from vial using tweezers and place vial on ice.
2. Remove rubber stopper from vial, then add 200 µL 1X PBS (1X), pH 7.4 to the vial.
3. Replace the rubber stopper, then vortex the tube to mix.
4. Transfer reconstituted sample to a 1.5-ml tube, then store on ice or at 4°C.

   Note: Store the reconstituted control at 4°C for up to 48 hours. For long term storage, store the reconstituted control at –80°C to –20°C for up to 4 months. Mix well to resuspend before use.

The final concentration of the control is 5×10^6 copies/µL.

Set up the processing plates

Set up the processing plates outside the instrument according to the following table. Cover the plates with a temporary seal, then store at room temperature for up to 1 hour while you set up Sample Plate.

Plate type	Plate position	Plate ID	Reagent	Volume per well
Deep well[1]	2	Wash 1 Plate	Wash Buffer	1000 µL
	3	Wash 2 Plate	80% Ethanol	1000 µL
	4	Wash 3 Plate	80% Ethanol	500 µL
	5	Elution Plate	Elution Solution	60 µL[2]
Standard[3]	6	Tip Comb	96DW Tip Comb	—

[1] KingFisher™ Deepwell 96 Plate
[2] The elution volume can be increased to a maximum of 100 µL.
[3] KingFisher™ 96 KF microplate

Set up Sample Plate, then start processing

(*Optional*) Reconstitute TaqMan® Universal Extraction Control Organism (*B. atrophaeus*) before use in step 3 (see "Reconstitute TaqMan® Universal Extraction Control Organism (B. atrophaeus)" on page 16).

1. Swirl the bottle of Enzyme Mix, then place on ice.
2. Add 50 µL of Enzyme Mix to each well in a KingFisher™ Deepwell 96 Plate (Sample Plate).

3. Add samples and controls to the appropriate well containing Enzyme Mix.

Sample or control	Instructions
Sample	Add 200–400 µL of sample to a well.
Negative Extraction Control (NEC)	Add 200–400 µL of Universal Transport Media to a well.
(*Optional*) TaqMan® Universal Extraction Control Organism (*B. atrophaeus*)	• Combine 10 µL of reconstituted control with 390 µL of Universal Transport Media in a well. *or* • Add 10 µL of reconstituted control to one or more sample wells.

4. On the KingFisher™ Flex instrument, select the **MVP_Ultra_Flex** script, then press **Start**.

5. Follow the instrument prompts to load sample and processing plates, then press **Start**.

Proceed immediately to the next step.

Continue processing to bind, wash, and elute the nucleic acid

1. During the enzyme treatment incubation on the instrument, prepare the Binding/Bead Mix.
   a. Vortex the tube of Nucleic Acid Binding Beads to fully resuspend the beads.
   b. Combine the following components for the required number of samples, plus 10% overage.

   IMPORTANT! Binding Solution is viscous. Pipet slowly to avoid bubbles and to ensure that the correct volume is delivered.

Component	Volume per sample
Binding Solution	530 µL
Nucleic Acid Binding Beads	20 µL
(*Optional*) TaqMan® Universal RNA Spike In/Reverse Transcription (Xeno) Control	10 µL
Total	**550 µL** or **560 µL**

2. Gently invert the Binding/Bead Mix 5 times to mix, then store at room temperature until the next step.
3. When prompted by the instrument (approximately 20 minutes after the start of the script), remove the Sample Plate from the instrument.
4. Add 10 µL of Proteinase K to each sample in the Sample Plate.

   Note: Add the Proteinase K to the sample separately from and before the Binding/Bead Mix. Combining the reagents, or adding in a different order can affect nucleic acid recovery.
5. Gently invert the Binding/Bead Mix 5 times to mix, then use a manual pipet (single or multi channel) to dispense the appropriate volume to each sample and control well in the Sample Plate.
   - 550 µL: Binding/Bead Mix only *or*
   - 560 µL: Binding/Bead Mix + TaqMan® Universal RNA Spike In/Reverse Transcription (Xeno) Control

   IMPORTANT! Binding/Bead Mix is viscous. Pipet slowly to avoid bubbles and to ensure that the correct volume is delivered. Invert the Binding/Bead Mix regularly to avoid bead settling.
6. Return Sample Plate to the instrument, then press **Start** to resume the script.
7. When processing is complete (~30 minutes after adding Binding/Bead Mix), remove Elution Plate from instrument.
   The purified nucleic acid is in Elution Plate.
8. Transfer the nucleic acid samples to a 96-well storage plate or seal Elution Plate.

Store nucleic acid samples on ice for immediate use or at –20°C for longer-term storage.

3 Perform preamplification and real-time PCR

Good laboratory practices for PCR and RT-PCR

- Wear clean gloves and a clean lab coat.
  - Do not wear the same gloves and lab coat that you have previously used when handling amplified products or preparing samples.
- Change gloves if you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation and reaction setup.
  - Amplification and analysis of products.
- Do not bring amplified products into the reaction setup area.
- Open and close all sample tubes carefully. Avoid splashing or spraying samples.
- Keep reactions and components capped as much as possible.
- Use a positive-displacement pipettor or aerosol-resistant barrier pipette tips.
- Clean lab benches and equipment periodically with 10% bleach solution or DNA decontamination solution.

Perform preamplification

Note: Preamplification of the TaqMan® Respiratory Tract Microbiota Amplification Control is not recommended.

1. Prepare PreAmp Reaction Mix: Combine the following components for the number of required reactions plus 10% overage, then mix thoroughly by pipetting up and down.

Component	Volume per reaction
TaqPath™ 1-Step RT-qPCR Master Mix, CG	2.5 µL
TaqMan® PreAmp Pool, Respiratory Tract Microbiota, 4X	2.5 µL

2. Distribute PreAmp Reaction Mix, then Nuclease-free Water or sample nucleic acid to the appropriate wells of a 96-well plate.

Component	Sample reaction	No-template control (NTC) reaction
PreAmp Reaction Mix	5 µL	5 µL
Sample DNA or NEC	5 µL	—
Nuclease-free Water	—	5 µL
Total volume per reaction	**10 µL**	**10 µL**

3. Seal the plate with adhesive film.

4. Gently vortex the plate for 10 seconds to mix, then briefly centrifuge to bring contents to the bottom of the wells.

5. Place the plate in a thermal cycler that is programmed with the following thermal cycling conditions, then start the run.

Stage	Step	Temperature	Time
Hold	UNG incubation[1]	25°C	2 minutes
Hold	Reverse transcription	50°C	30 minutes
Hold	Activation	95°C	2 minutes
Cycling (14 cycles)	Denaturation	95°C	15 seconds
	Annealing/ Extension	60°C	2 minutes
Hold	Inactivation	99.9°C	10 minutes
Hold	—	4°C	Hold

[1] Heat-labile UNG is completely inactivated during the initial ramp to 95°C.

6. Store the plate on ice until dilution for PCR (see "Dilute the preamplified product" on page 21).

Dilute the preamplified product

To determine dilution volumes, first determine the total volume of diluted preamplified sample that is required for PCR (see Chapter 4, "Prepare and run TaqMan® Array Cards"). We recommend that you prepare only the volume of diluted preamplified sample that is required for your experiment. The undiluted preamplified sample can be stored at –20°C long term.

1. Vortex, then briefly centrifuge the plate that contains the completed preamplification sample reactions.

2. Remove the adhesive film from the plate.

3. Prepare a 1:20 dilution of the preamplified samples in a new 96-well plate.
   a. Transfer the desired volume of the preamplified samples to a new 96-well plate (for example, 2 µL).
   b. Add the appropriate volume of Nuclease-free Water to each sample and control well (for example, 38 µL).
4. Seal the plate with new adhesive film.
5. Vortex the plate for 10 seconds, then briefly centrifuge.
6. Proceed directly to PCR (see Chapter 4, "Prepare and run TaqMan® Array Cards").

Seal the plate that contains the unused portion of the undiluted preamplified product, then store at –20°C.

4 Prepare and run TaqMan® Array Cards

For detailed instructions for handling TaqMan® Array Cards, see Appendix C, "Detailed procedures for preparation of a TaqMan® Array Card".

Download the plate file for the array card at **thermofisher.com/taqmanfiles**.

1. Allow the card to equilibrate to room temperature.
2. Gently mix the bottle of TaqMan® Fast Advanced Master Mix, No UNG.

   IMPORTANT! Ensure that you use TaqMan® Fast Advanced Master Mix, No UNG. If you use a master mix that contains UNG, the preamplified samples will be degraded.
3. For each port, combine the following components.

   (*Optional*) Use 10 µL of TaqMan® Respiratory Tract Microbiota Amplification Control plus 10 µL of Nuclease-free Water instead of diluted preamplified product, as a positive amplification control sample.

Component	Volume per port
Diluted preamplified product	20 µL
TaqMan® Fast Advanced Master Mix, No UNG	50 µL
Nuclease-free Water	30 µL
Total volume per port	**100 µL**

4. Fill each port with 100 µL of prepared mix.
5. Centrifuge the card at 1,200 rpm (301 × *g*) for 1 minute.
6. Repeat step 5.
7. Seal the card using TaqMan® Array Card Sealer (see "Seal the card" on page 42 for detailed instructions).
8. Load the card into the real-time PCR instrument, then set up the experiment in the instrument software.
   - Experiment type—**Array Card**
   - Experiment—**Standard curve**
   - Run type—**Fast**
   - Sample and assay assignments—Import the plate file (TXT) for the card, then assign samples.

- Run method—Change the default run method to the following settings:

Step	Stage	Cycles	Temp.	Time
Activation	1	1	95°C	10 minutes
Amplification	2	40	95°C	3 seconds
			60°C	30 seconds

9. Run the program.

5 Analyze data

Select analysis software

Software	Analysis option	
	C_{rt} analysis	QC metrics
Relative Quantification App (recommended)[1] Access the app at thermofisher.com/connect.	✓	✓
QuantStudio™ 12K Flex Software	✓	✓
QuantStudio™ 6 and 7 Flex Software	✓	✓ [2]

[1] To perform data analysis using the app, you must export your data. For detailed instructions about exporting data, see the instrument documentation].
[2] QC metrics are not available v1.3 or earlier.

Review results

1. In the analysis settings of the software, select the relative C_{rt} method.
   The relative C_{rt} method is recommended for dried-down assays. Dried-down assays can reconstitute at different rates, causing a dip in the early cycles of the baseline. C_{rt} can correct for a variable baseline.
   - In RQ App:
     a. Click **(Analysis Settings)**.
     b. In the **C_q Settings** tab, select **Use C_{rt}**.
     c. In the **Endogenous controls** tab, select **Skip target normalization**.
     d. Click **Finish**.
   - In QuantStudio™ 12K Flex Software or QuantStudio™ 7 Flex Software—select **Analysis Settings ▸ Ct Settings ▸ Algorithm Settings ▸ Relative Threshold**.
2. Review amplification curves (in log or linear view), C_{rt} values, and amplification curve QC metrics (Amp Score and C_q Confidence) for each reaction.

QC metric	Description
Amp Score	A value to indicate the quality of the amplification curve.
C_q Confidence	A value to reflect the reliability of the derived C_q.

3. (*Optional*) Filter data in the order indicated in the following table.

   Note: We encourage testing and establishing your own C_{rt} cut-off value and amplification curve QC metrics for each assay to achieve high sensitivity and specificity.

Parameter to examine	Consider filtering out sample data using the following cut-off values
C_{rt}	$C_{rt} > 30$
Amp Score	Amp Score < 1.2 **Note:** The RNase P (RPPH1) assay has a cut-off value of < 1.0
C_q Confidence	C_q Confidence < 0.7 **Note:** The following assays have a cut-off value of < 0.5 • RNase P (RPPH1) • Human Rhinovirus 1/2 (RV_1of2) • Human Rhinovirus 2/2 (RV_2of2) • Bordetella holmesii (B. holmesii)

Note: The C_{rt} method is recommended for analysis. If you select the comparative C_t method, use the following analysis settings. Review real-time data as described above.
- Baseline—Auto
- Threshold—0.2 C_t

Considerations for data analysis

Organisms that are covered by more than one assay

For full strain coverage of adenoviruses or rhinoviruses, two assays are used. A positive result with either or both of the assays indicates a positive result for the organism.

Species-specific assays that are also covered by pan or broad coverage assays

Species-specific assay	Considerations for data analysis
Flu A assays	• The Flu_A_pan assay detects Influenza A H1 and H3, for which there are also strain-specific assays. • Samples that are positive for the Flu_A_H1 or Flu_A_H3 assay typically are positive for the Flu_A_pan assay.
Bordetella assays	• The Bordetella assay detects *B. pertussis*, *B. bronchiseptica*, and *B. parapertussis* strains. Strain-specific assays for *B. pertussis* and *B. holmesii* are also available. • Most samples that are positive for the *B. pertussis* assay are also positive with the Bordetella assay.

Species-specific assay	Considerations for data analysis
RSV assays	• Samples that are positive for the RSVA assay may be detected at a lower efficiency (a difference of several C_{rt} values) by the RSVB assay.
Enterovirus (EV) and rhinovirus (RV) assays	• The RV assays detect both RV and EV strains whereas the EV assays are specific for EV strains. Thus enterovirus positive samples are detected by both EV and RV assays whereas rhinovirus positive samples are detected only by the RV assays. • The EV_pan assay detects all human enterovirus species except D68, for which there is a strain-specific EV_D68 assay. • Samples that are positive for the EV_D68 assay may be detected at a lower efficiency (a difference of several C_{rt} values) by the EV_pan assay.
Bacterial and HHV assays	• It is not unusual to detect *M. catarrhalis*, *H. influenzae*, *K. pneumoniae*, *S. pneumoniae*, and *S. aureus* in respiratory samples as these are commensal or transiently commensal upper respiratory tract microbes. • Due to the high prevalence of human infection with HHV4 (EBV) and HHV6 viruses, these viruses can be detected at low levels in some respiratory samples.



Troubleshooting

Troubleshooting: Nucleic Acid Isolation

Observation	Possible cause	Recommended action
Beads remain in sample after elution	Excessive amount of recovered host genomic DNA/RNA is preventing nucleic acid separation from the beads.	Increase elution volume to 100 µL.
		Reduce the input volume of starting sample to 200 µL.
Reduced extraction efficiency of TaqMan® Universal RNA Spike In/Reverse Transcription (Xeno) Control	Proteinase K enzyme was either omitted from the sample or added incorrectly.	Always add Proteinase K enzyme to the sample separately and before adding the Binding/Bead Master mix.
	TaqMan® Universal RNA Spike In/Reverse Transcription (Xeno) Control added at the wrong step.	Ensure that theTaqMan® Universal RNA Spike In/Reverse Transcription (Xeno) Control is added to the Binding/Bead Master mix before dispensing into sample wells.

Troubleshooting: After removing the card from packaging

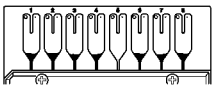
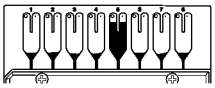
Observation	Possible cause	Recommended action
Water condenses on the reaction wells (optical side of the card)	The card was not at room temperature before being removed from the packaging.	Remove condensation on the reaction wells by lightly blowing room temperature pressurized nitrogen or an air blower on the wells. **IMPORTANT!** Ensure that all water condensation is removed. The optical side of the card must be free of water condensation.

Troubleshooting: After loading PCR reaction mix into the card

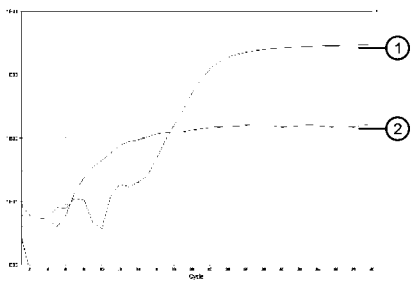
Observation	Possible cause	Recommended action
Fill reservoirs have bubbles in the PCR reaction mix	When loading the card with PCR reaction mix, air was introduced into the fill reservoir.	Inspect the affected rows after centrifuging and sealing the card. Note wells that contain bubbles, then consider omitting these wells from analysis.
Fill reservoir is not full of PCR reaction mix	The PCR reaction mixture was not correctly pipetted into the fill reservoir.	Be sure to correctly pipette the entire PCR reaction mixture (100 µL) into the fill reservoir. Add more sample-specific PCR reaction mix to the fill reservoir.

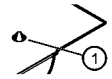
Observation	Possible cause	Recommended action
PCR reaction mix leaks from the vent port into the fill reservoir	The PCR reaction mixture was not correctly pipetted into the fill reservoir.	Be sure to correctly pipette the entire PCR reaction mixture (100 µL) into the fill reservoir. Add more sample-specific PCR reaction mix to the fill reservoir.

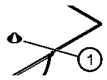
Troubleshooting: After centrifuging the card

Observation	Possible cause	Recommended action
Fill reservoir completely empty 	Some wells were filled improperly.	Continue with running the card. However, consider omitting the wells associated with that fill reservoir.
PCR reaction mix remains in a fill reservoir 	Though rare, the fill port is blocked.	Inspect the card for blocked fill port or a pinched channel. If the fill reservoir is defective, contact Support.
	Filling is incomplete or not consistent.	Centrifuge the card again for 1 minute.
		If the filling is still incomplete after the additional centrifuge cycle, continue with running the card. However, consider omitting the wells associated with that fill reservoir.

Troubleshooting: After running the card and reviewing run results

Observation	Possible cause	Recommended action
Unexpected C_{rt} values in the amplification plot  ① Expected C_{rt} value (noted in most replicates) ② Unexpected C_{rt} value (too low)	Unexpectedly low C_{rt} values (< 10) — Signal variation or noise in early PCR cycles	Review amplification curves, Amp Score, and C_q Confidence values.
		Consider filtering C_{rt} values from analysis.
		Compare replicates, if available.
		Dilute the samples, then repeat the experiment.
	Unexpectedly high C_{rt} values (27–30) — Sporadic amplification	Review amplification curves, Amp Score, and C_q Confidence values.
		Compare replicates, if available.
		Repeat the experiment.

Observation	Possible cause	Recommended action
No amplification in some wells	Empty wells due to improper card sealing.	When sealing the card, use a slow and steady motion to push the carriage across the TaqMan® Array Card Sealer. **IMPORTANT!** Do not move the carriage back across the card. See "Seal the card" on page 42.
	Positive sample is not present in the wells.	No action required. Consider using one or more positive controls to confirm nucleic acid recovery.
No amplification for portions of the card	The card was misaligned in the block during the instrument run.	Inspect the card for crushed or distorted feet. If there are damaged feet, contact Support.  ① Array card feet
	Positive sample is not present in the wells.	No action required. Consider using one or more positive controls to confirm nucleic acid recovery.
No amplification within or across one or more rows	Empty wells due to improper card sealing.	When sealing the card, use a slow and steady motion to push the carriage across the TaqMan® Array Card Sealer.
	Empty wells due to misalignment of the TaqMan® Array Card Sealer.	If the TaqMan® Array Card Sealer is misaligned, contact Support.
	PCR reaction mix improperly prepared.	Ensure that all reaction components were added to the PCR reaction mix.
	Positive sample is not present in the wells.	No action required. Consider using one or more positive controls to confirm nucleic acid recovery.

Observation	Possible cause	Recommended action
The baseline is variable	The dried-down assays on the card were reconstituted at different rates, causing a dip in the early cycles of the baseline.	Use the relative threshold algorithm (C_{rt}). C_{rt} can correct for a variable baseline.
		Use the Relative Quantification application, available on the Connect. The Relative Quantification application uses C_{rt} if the software specific to your instrument does not have the relative threshold algorithm.
Noise in the amplification plots for portions of the card	The card was misaligned in the block during the instrument run.	Inspect the card for crushed or distorted feet. If there are damaged feet, contact Support.  ① Array card feet
A gradient signal across the card	The card was in a diagonal position during centrifugation because not all of the positions in the card holder were filled.	Repeat the assay with a new card. Ensure that all the positions in the centrifuge card holder are filled.

1-step RT-PCR procedure

For maximum sensitivity, we strongly recommend using the 2-step procedure as described in Chapter 3, "Perform preamplification and real-time PCR".

The following 1-step procedure can be used if sensitivity requirements can be met without using preamplification.

Note: This 1-step procedure has not been fully optimized for respiratory tract microorganism detection.

Prepare and run TaqMan® Array Cards (alternate 1-step RT-PCR procedure)

The same thermal cycling program can be used for both RNA and DNA targets. The reverse transcription step does not affect performance with DNA targets.

For detailed instructions for handling TaqMan® Array Cards, see Appendix C, "Detailed procedures for preparation of a TaqMan® Array Card".

1. Allow the card to equilibrate to room temperature.
2. Gently mix the bottle of TaqPath™ 1-Step RT-qPCR Master Mix, CG.
3. For each port, combine the following components.

Component	Volume per port
Nucleic acid[1]	50 µL
TaqPath™ 1-Step RT-qPCR Master Mix, CG	25 µL
Nuclease-free Water	25 µL
Total volume per port	**100 µL**

[1] For more information, see Chapter 2, "Isolate nucleic acid using MagMAX™ Viral/Pathogen Ultra Nucleic Acid Isolation Kit".

10–50 µL of nucleic acid sample can be used. The ratio of nucleic acid sample to water can be adjusted based on:

- Sample availability
- Need for sample archiving
- Sample splitting for port replicates

4. Fill each port with 100 µL of prepared mix.
5. Centrifuge the card at 1,200 rpm for 1 minute.

6. Repeat step 5.
7. Seal the card using TaqMan® Array Card Sealer.
8. Load the card into the real-time PCR instrument, then set up the experiment in the instrument software.

   Settings:

   - Experiment type—**Array Card**
   - Experiment—**Standard Curve**
   - Run type—**Fast**
   - Sample and assay assignments—Import the plate file (TXT) for the card, then assign samples
   - Run method—Change the default run method to the following settings:

Step	Stage	Cycles	Temperature	Time
UNG incubation[1]	1	1	25°C	2 minutes
Reverse transcription	2	1	50°C	15 minutes
RT inactivation/denaturation	3	1	95°C	2 minutes
Amplification	4	40	95°C	3 seconds
			60°C	1 minute[2]

[1] Heat-labile UNG is completely inactivated during the initial ramp to 95°C.
[2] For sample volumes < 30 µL, adjust the time to 30 seconds.

9. Run the program.

Data analysis parameters for 1-step RT-PCR

We encourage testing and establishing your own C_{rt} cut-off value and amplification curve QC metrics for each assay to achieve high sensitivity and specificity.

Parameter to examine	Consider filtering out sample data using the following cut-off values
C_{rt}	$C_{rt} > 35$
Amp Score	Amp Score < 1.2 **Note:** The RNase P (RPPH1) assay has a cut-off value of < 1.0
C_q Confidence	C_q Confidence < 0.7 **Note:** The following assays have a cut-off value of < 0.5 • RNase P (RPPH1) • Bordetella pertussis (B. pertussis) • Bordetella holmesii (B. holmesii)



For more information about reviewing the results, see the following sections:

- "Review results" on page 25
- "Considerations for data analysis" on page 26



Detailed procedures for preparation of a TaqMan® Array Card

- TaqMan® Array Card overview 36
- Guidelines for preparation of a card 37
- TaqMan® Array Card diagram 38
- Load the PCR reaction mix 39
- Centrifuge the card 39
- Seal the card 42

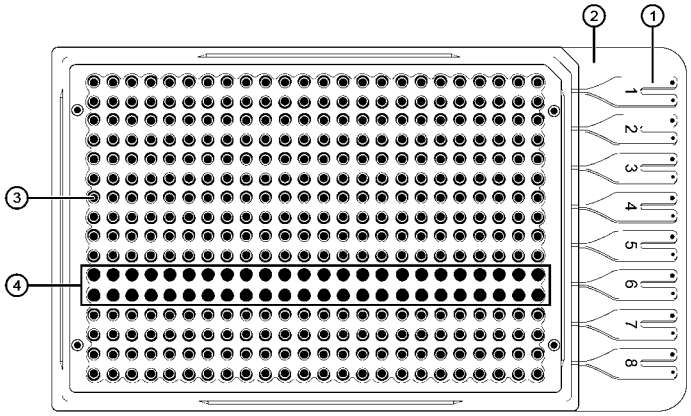
TaqMan® Array Card overview

TaqMan® Array Cards are 384-well microfluidic cards that are prepared with dried-down TaqMan® Assays.

Advantages of using TaqMan® Array Cards include:

- Small-volume design that minimizes sample and reagent consumption.
- Streamlined reaction setup that saves time and reduces labor-intensive steps.
- Access to high-throughput, 384-well format without liquid-handling robotics.
- Two-fold discrimination detection at the 99.7% confidence level.
- Standardization across multiple samples in multiple laboratories.

Each card can run 1 to 8 samples against 12 to 384 TaqMan® Assay targets (including controls).



① **Fill reservoir**—Each reservoir is loaded with a sample-specific PCR reaction mix; the associated reaction wells fill with that sample (8 total reservoirs)
② **Fill reservoir strip**—Support strip for fill reservoirs; removed before running the card
③ **Reaction well**—Each well contains dried-down assay (384 total reaction wells)
④ **Reaction well row**—A set of reaction wells that fill with the same sample-specific PCR reaction mix (8 total rows, each row associated with a single fill reservoir)

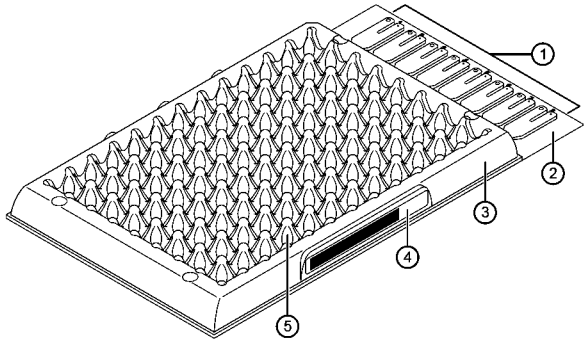
Guidelines for preparation of a card

- Keep the card protected from light and stored as indicated until ready for use. Excessive exposure to light may affect the fluorescent probes of the dried-down assays in the card.
- Before removing the card from its packaging:
  - Prepare each sample-specific PCR reaction mix.
  - Allow the card to reach room temperature.
- Load each fill reservoir with 100 µL of sample-specific PCR reaction mix.
  - Each fill reservoir contains a single sample as determined by the card layout.
  - The 100–µL volume ensures adequate filling of each reaction well. Volumes smaller than 100 µL result in insufficiently filled cards.
- Do not allow the micropipette tip to contact the coated foil beneath the fill port.

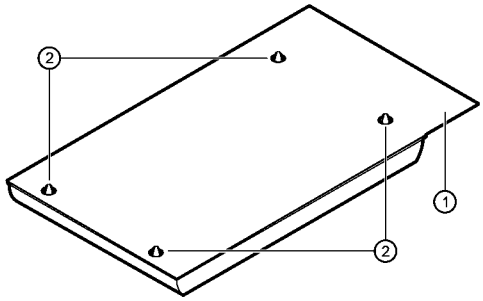
- Load the card with PCR reaction mix *before* centrifuging the card.
  During centrifugation, the PCR reaction mix resuspends the dried-down assays in each well of the card. Adding sample after centrifuging disrupts the assay layout of the card.
- Run the card within the time allowed by the Master Mix.
- If the card is not run immediately, protect it from light and store at 2-8°C.

TaqMan® Array Card diagram

A TaqMan® Array Card includes 8 fill reservoirs and 384 reaction wells.

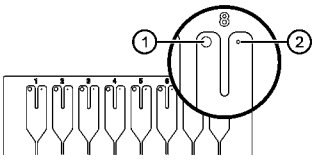


① Fill reservoirs (8 total)
② Fill reservoir strip
③ Array card carrier
④ Array card barcode
⑤ Reaction well (384 total)



① Foil
② Array card feet

The fill reservoir includes a fill port and a vent port. Use the fill port to load PCR reaction mix into the card.



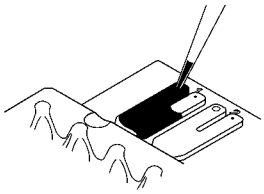
① Fill port
② Vent port

Load the PCR reaction mix

Before removing the card from its packaging:

- Prepare each sample-specific PCR reaction mix.
- Allow the card to reach room temperature.

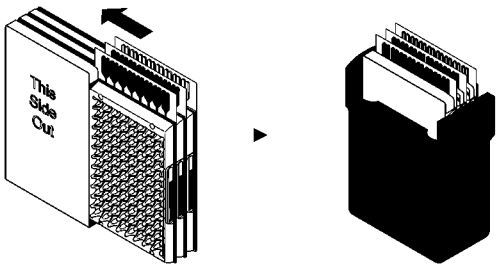
1. Carefully remove the card from its packaging.
2. Place the card on the benchtop with its foil-side down.
3. Load 100 µL of the sample-specific PCR reaction mix into a micropipette.
4. Hold the micropipette in an angled position, then place the tip into a fill port of the card.
5. Slowly dispense the entire volume of reaction mix so that it sweeps in and around the fill reservoir toward the vent port.



Centrifuge the card

1. Load the cards into the centrifuge buckets.
   a. Place the bucket on the benchtop with its label facing the front of the bench.
   b. Insert the cards into the card holder, ensuring that:
      - The fill reservoirs extend upwards out of the card holder.
      - The reaction wells face the label-side of the card holder.
   c. Insert blank balance cards into any empty positions of the card holder. All three positions in the card holder must be filled.

d. Place the loaded card holder into the bucket so that the card holder label faces the front of the bucket.



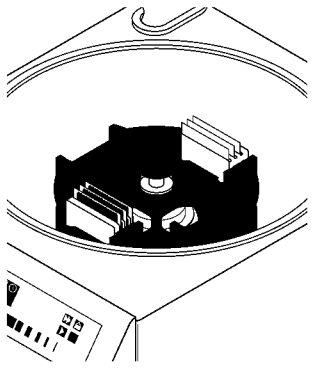
2. Configure the centrifuge using its front-panel controls.
   a. Set the bucket type to **15679**.
   b. Set the following parameters according to the control panel type.

Parameter	EASYSet (touchpad-operated)	QUIKSet (knob-operated)
Increasing ramp rate	9	3
Decreasing ramp rate	9	—
Rotational speed	1,200 rpm (331 × *g*)	1,200 rpm
Centrifugation time [1]	1 minute	1 minute

[1] You will centrifuge the cards twice, each time for 1 minute (see step 4).

IMPORTANT! A speed that is set too high can deform the card.

3. Load the buckets into the centrifuge.
   a. Press ⏏ on the centrifuge control panel to open the centrifuge cover.
   b. Place each loaded bucket onto an open rotor arm of the centrifuge.
      Ensure that each bucket can swing easily within its slotted position on the rotor arm.
   c. If there are empty rotor arms, prepare buckets with blank balance cards as described in step 1. Place the balance buckets onto the rotor arms.
      The rotor must be evenly loaded and opposing buckets must hold the same weight.



Centrifuge is properly loaded and balanced.

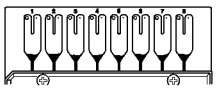
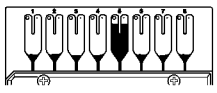
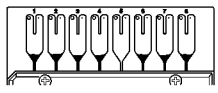
d. Close the centrifuge cover.

4. Run the centrifuge.
   a. Press ▶.
      The centrifuge will start, then automatically stop after 1 minute.
   b. Repeat substep 4a so that the cards are centrifuged for a total of two, consecutive, 1-minute centrifugations.

   IMPORTANT! Do not centrifuge the cards continuously for 2 minutes. The ramping up in speed during the *two, consecutive 1-minute* centrifugations is important for proper filling.

5. Remove the cards from the centrifuge.
   a. Press ⏏.
   b. Remove the buckets from the centrifuge, then remove the card holders from the buckets.
   c. Remove each card from the card holder by lifting it gently by the card carrier sides.

6. Examine the cards for proper filling.
   When properly filled, the remaining volumes of PCR reaction mix are consistent reservoir to reservoir. If the card is not properly filled, see Appendix A, "Troubleshooting".

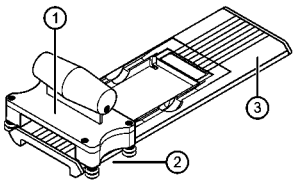
Consistent filling	Inconsistent filling	
		

Seal the card

The TaqMan® Array Card Sealer isolates the wells of an array card after it is loaded with PCR reaction mix and centrifuged. The sealer uses a precision stylus assembly (under the carriage) to seal the main fluid distribution channels of the array card.

Note: In some documents, the TaqMan® Array Card Sealer is referred to as a Stylus Staker.

1. Position the TaqMan® Array Card Sealer and its carriage before inserting a card.
   a. Place the sealer on a benchtop that is approximately waist-high so that the carriage can be easily maneuvered.
   b. Position the sealer with the carriage starting position toward the front of the bench.
   Ensure that the engraved arrows on the sealer point away from you.

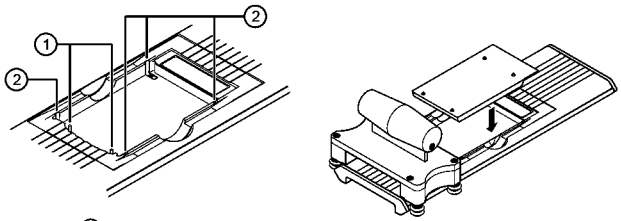


① Carriage
② Carriage starting position
③ Carriage ending position

   c. Ensure that the carriage for the sealer is at the starting position.

IMPORTANT! Do not insert a filled card into the sealer if the carriage is *not* in its starting position. The card will be irreparably damaged if the carriage is moved backwards across the card towards the starting position.

2. Insert a filled, centrifuged card into the sealer.
   a. Hold the card with its foil-side up.
   b. Orient the card over the sealer with the fill reservoirs of the card toward the ending position.
   c. Align the rear pin grooves of the card to the alignment pins of the sealer.

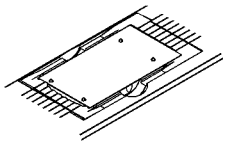


① Alignment pins
② Spring clips

   d. Gently place the card on top of the sealer.

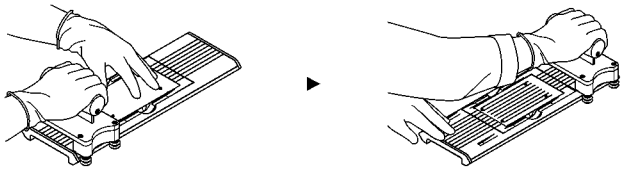
e. Gently push the card until it is seated securely in the sealer.

When properly seated, the foil surface of the card is level with the base of the sealer and the spring clips hold the card securely in place.



3. Slowly and steadily push the carriage across the sealer in the direction of the engraved arrows.

Push the carriage across the entire length of the card until the carriage meets the mechanical stops at the ending position.



IMPORTANT!

- Do not use excessive force or speed when pushing the carriage across the card.
- Do not move the carriage back across the card. Leave the carriage at the ending position while removing the card from the sealer.

4. Remove the sealed card from the sealer by grasping the sides of the card and lifting it off.

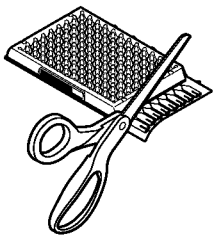
Use the thumb slot in the middle of the sealer to access the card.

5. Examine the card for proper sealing.

Note: When properly sealed, the indentations from the sealer carriage align with the main channels of the card.

If the indentations do not align or if the foil is damaged, do not use the card.

6. Use scissors to trim the fill reservoir strip from the card. Use the edge of the card carrier as a guide.



IMPORTANT! Completely remove the fill reservoir strip. Any remaining plastic that extends beyond the card edge can prevent the card from seating properly on the sample block and can affect amplification.

Correct trim	Incorrect trim
	

The card is now ready to run on the instrument.

Run the card within the time allowed by the Master Mix.



Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2009-P.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

Documentation and support

Related documentation

Document	Publication Number
TaqMan® Respiratory Tract Microbiota Amplification Control Product Information Sheet	MAN0018533
TaqMan® Universal RNA Spike In/Reverse Transcription (Xeno) Control Product Information Sheet	MAN0018534
QuantStudio™ 12K Flex Real-Time PCR System: Multi-Well Plates and Array Card Experiments User Guide	4470935
QuantStudio™ 12K Flex Real-Time PCR System Maintenance and Administration Guide	4470689
QuantStudio™ 6 and 7 Flex Real-Time PCR Systems Maintenance and Administration Guide	4489821
Thermo Scientific™ KingFisher™ Flex User Manual	N07669
TaqPath™ 1-Step RT-qPCR Master Mix, CG User Guide	MAN0007959

Symbols that may be displayed on the instrument, in the software, or in this guide

Symbol	Description	Symbol	Description
	MANUFACTURER		DATE OF MANUFACTURE
REF	CATALOG NUMBER	SN	SERIAL NUMBER
	CONSULT INSTRUCTIONS FOR USE[1]		CAUTION[1]

[1] "Documentation and support" on page 48

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  - Product FAQs
  - Software, patches, and updates
  - Training for many applications and instruments
- Order and web support
- Product documentation
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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appliedbiosystems PRODUCT INFORMATION SHEET

TaqMan® Array Respiratory Tract Microbiota Comprehensive Card

Catalog Number A41238

Pub. No. MAN0018632 **Rev.** A.0

Note: For safety and biohazard guidelines, see the "Safety" appendix in the *Respiratory Tract Microbiota Profiling Experiments using TaqMan® Array Cards Application Guide* (Pub. No. MAN0017951). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Product description

TaqMan® Array Respiratory Tract Microbiota Comprehensive Card is an efficient, easy-to-use TaqMan® Array Card for the characterization of key respiratory tract microbes. The array card includes TaqMan® assays that have been optimized for detection of 42 respiratory tract viral, bacterial, and fungal microbes. The array card also includes control assays for TaqMan® Universal Extraction Control Organism (*B. atrophaeus*), TaqMan® Universal RNA Spike In/Reverse Transcription (Xeno) Control, the human RNase P RPPH1 gene, and the human 18S ribosomal RNA gene. For a complete list of assays included in the TaqMan® Array Respiratory Tract Microbiota Comprehensive Card, see Table 1.

The assays perform well with total nucleic acid that is isolated from nasopharyngeal swab, nasopharyngeal aspirate, and bronchoalveolar lavage (BAL) samples using the MagMAX™ Viral/Pathogen Ultra Nucleic Acid Isolation Kit (Cat. No. A42356).

TaqMan® assay designs and assay target sequences have undergone rigorous bioinformatics selection and analysis to maximize strain coverage and minimize potential for off-target cross-reactivity. Qualified TaqMan® assays also undergo performance testing to ensure that results are accurate and reproducible with high levels of sensitivity and specificity.

See "Related documentation" on page 2 for resources that contain detailed information about using the TaqMan® Array Respiratory Tract Microbiota Comprehensive Card.

Contents and storage

Component	Amount	Storage
TaqMan® Array Respiratory Tract Microbiota Comprehensive Card	10 cards[1]	2°C to 8°C

[1] Minimum order.

TaqMan® assays for respiratory tract microbiota profiling

The following assays are included in the TaqMan® Array Respiratory Tract Microbiota Comprehensive Card.

Table 1 Assays for respiratory tract microbiota targets

Target organism	Assay name	Nucleic acid type	Assay ID
Bacteria			
Bordetella bronchiseptica / parapertussis / pertussis	Bordetella	DNA	Ba06439624_s1
Bordetella holmesii	B.holmesii	DNA	Ba06439621_s1
Bordetella pertussis	B.pertussis	DNA	Ba06439623_s1
Chlamydophila pneumoniae	C.pneumoniae	DNA	Ba06439616_s1
Coxiella burnetii	C.burnetii	DNA	Ba06439618_s1
Haemophilus influenzae	H.influenzae	DNA	Ba06439625_s1
Klebsiella pneumoniae	K.pneumoniae	DNA	Ba04932083_s1
Legionella pneumophila	L.pneumophila	DNA	Ba06439617_s1
Moraxella catarrhalis	M.catarrhalis	DNA	Ba06439622_s1
Mycoplasma pneumoniae	M.pneumoniae	DNA	Ba06439620_s1
Staphylococcus aureus	S.aureus	DNA	Ba04646259_s1
Streptococcus pneumoniae	S.pneumoniae	DNA	Ba06439619_s1
Fungus			
Pneumocystis jirovecii	P.jirovecii	DNA	Fn06439626_s1

Target organism	Assay name	Nucleic acid type	Assay ID
Virus			
Adenovirus	AdV_1of2	DNA	Vi99990001_po
Adenovirus	AdV_2of2	DNA	Vi99990002_po
Human Bocavirus	HBoV	DNA	Vi99990003_po
Human Coronavirus 229E	CoV_229E	RNA	Vi06439671_s1
Human Coronavirus HKU1	CoV_HKU1	RNA	Vi06439674_s1
Human Coronavirus NL63	CoV_NL63	RNA	Vi06439673_s1
Human Coronavirus OC43	CoV_OC43	RNA	Vi06439646_s1
Human Enterovirus (pan assay)	EV_pan	RNA	Vi06439631_s1
Human Enterovirus D68	EV_D68	RNA	Vi06439669_s1
Human Metapneumovirus (hMPV)	hMPV	RNA	Vi99990004_po
Human Parainfluenza virus 1	hPIV1	RNA	Vi06439642_s1
Human Parainfluenza virus 2	hPIV2	RNA	Vi06439672_s1
Human Parainfluenza virus 3	hPIV3	RNA	Vi06439670_s1
Human Parainfluenza virus 4	hPIV4	RNA	Vi99990005_po
Human Parechovirus	HPeV	RNA	Vi99990006_po
Human Respiratory Syncytial Virus A (RSVA)	RSVA	RNA	Vi99990014_po
Human Respiratory Syncytial Virus B (RSVB)	RSVB	RNA	Vi99990015_po
Human Rhinovirus 1/2	RV_1of2	RNA	Vi99990016_po
Human Rhinovirus 2/2	RV_2of2	RNA	Vi99990017_po
Human herpesvirus 3 (HHV3 – Varicella zoster Virus)	HHV3	DNA	Vi06439647_s1
Human herpesvirus 4 (HHV4 – Epstein-Barr Virus)	HHV4	DNA	Vi06439675_s1
Human herpesvirus 5 (HHV5 – Cytomegalovirus)	HHV5	DNA	Vi06439643_s1
Human herpesvirus 6 (HHV6)	HHV6	DNA	Vi06439627_s1
Influenza A	Flu_A_pan	RNA	Vi99990011_po
Influenza A/H1-2009	Flu_A_H1	RNA	Vi99990009_po
Influenza A/H3	Flu_A_H3	RNA	Vi99990010_po
Influenza B	Flu_B_pan	RNA	Vi99990012_po
Measles virus	Measles	RNA	Vi99990013_po
Middle East Respiratory Syndrome coronavirus (MERS)	MERS_CoV	RNA	Vi06439644_s1
Mumps virus	Mumps	RNA	Vi06439657_s1
Severe Acute Respiratory Syndrome coronavirus (SARS)	SARS_CoV	RNA	Vi06439634_s1

Table 2 Assays for respiratory tract microbiota controls

Control name	Assay name	Nucleic acid type	Assay ID
TaqMan® Universal Extraction Control Organism (*B. atrophaeus*)	B.atrophaeus	DNA	Ba06596576_s1
TaqMan® Universal RNA Spike In/Reverse Transcription (Xeno) Control	Xeno	RNA	Ac00010014_a1
Human RNase P RPPH1 gene	RPPH1	DNA	Hs04930436_g1
Human 18S ribosomal RNA gene[1]	18S	DNA	Hs99999901_s1

[1] Human 18S ribosomal RNA is used as a manufacturing control for the array card and is not an assay specific to Respiratory Tract Microbiota profiling.

Related documentation

Document	Pub. No.
Respiratory Tract Microbiota Profiling Experiments using TaqMan® Array Cards Application Guide	MAN0017951
Respiratory Tract Microbiota Profiling Experiments using TaqMan® Array Cards Quick Reference	MAN0018528
Isolation of Nucleic Acid for Respiratory Tract Microbiota Profiling Experiments Quick Reference	MAN00018526

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Revision history: Pub. No. MAN0018632

Revision	Date	Description
A.0	27 August 2019	New document.

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APPENDIX 4

Essentials of Real Time PCR

About Real-Time PCR Assays

Real-time Polymerase Chain Reaction (PCR) is the ability to monitor the progress of the PCR as it occurs (i.e., in real time). Data is therefore collected throughout the PCR process, rather than at the end of the PCR. This completely revolutionizes the way one approaches PCR-based quantitation of DNA and RNA. In real-time PCR, reactions are characterized by the point in time during cycling when amplification of a target is first detected rather than the amount of target accumulated after a fixed number of cycles. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. In contrast, an endpoint assay (also called a "plate read assay") measures the amount of accumulated PCR product at the end of the PCR cycle.

About Sequence Detection Chemistries

Overview Applied Biosystems has developed two types of chemistries used to detect PCR products using Sequence Detection Systems (SDS) instruments:

- TaqMan® chemistry (also known as "fluorogenic 5′ nuclease chemistry")
- SYBR® Green I dye chemistry

TaqMan® Chemistry

The TaqMan chemistry uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR cycles.

Assay Types that Use TaqMan Chemistry

The TaqMan chemistry can be used for the following assay types:
Quantitation, including:

- One-step RT-PCR for RNA quantitation
- Two-step RT-PCR for RNA quantitation
- DNA/cDNA quantitation
- Allelic Discrimination
- Plus/Minus using an IPC

SYBR Green I Dye Chemistry

The SYBR Green I dye chemistry uses SYBR Green I dye, a highly specific, double-stranded DNA binding dye, to detect PCR product as it accumulates during PCR cycles.

The most important difference between the TaqMan and SYBR Green I dye chemistries is that the SYBR Green I dye chemistry will detect all double-stranded DNA, including non-specific reaction products. A well-optimized reaction is therefore essential for accurate results.

Assay Types that Use SYBR Green I Dye Chemistry

The SYBR Green I dye chemistry can be used for the following assay types:

- One-step RT-PCR for RNA quantitation
- Two-step RT-PCR for RNA quantitation
- DNA/cDNA quantitation

TaqMan Chemistry

Background

Initially, intercalator dyes were used to measure real-time PCR products. The primary disadvantage to these dyes is that they detect accumulation of both specific and nonspecific PCR products.

Development of TaqMan Chemistry

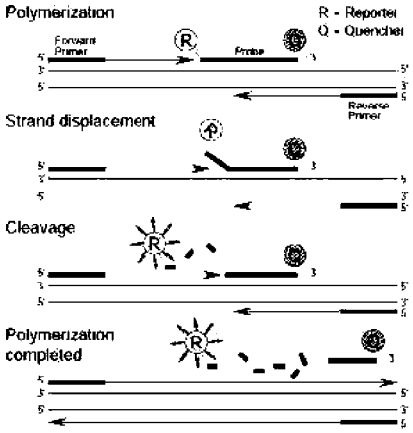
Real-time systems for PCR were improved by the introduction of fluorogenic-labeled probes that use the 5′ nuclease activity of Taq DNA polymerase. The availability of these fluorogenic probes enabled the development of a real-time method for detecting only specific amplification products. The development of fluorogenic labeled probes also made it possible to eliminate post-PCR processing for the analysis of probe degradation

How TaqMan Sequence Detection Chemistry Works

The TaqMan chemistry uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR. Here's how it works:

Step Process

1. An oligonucleotide probe is constructed containing a reporter fluorescent dye on the 5′ end and a quencher dye on the 3′ end. While the probe is intact, the proximity of the quencher dye greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (FRET) through space.
2. If the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by the 5′ nuclease activity of Taq DNA polymerase as this primer is extended.
3. This cleavage of the probe:
   - Separates the reporter dye from the quencher dye, increasing the reporter dye signal.
   - Removes the probe from the target strand, allowing primer extension to continue to the end of the template strand. Thus, inclusion of the probe does not inhibit the overall PCR process.
4. Additional reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescence intensity proportional to the amount of amplicon produced.



Two Types of TaqMan® Probes

Applied Biosystems offers two types of TaqMan probes:

- TaqMan® probes (with TAMRA™ dye as the quencher dye)
- TaqMan® MGB probes

TaqMan® MGB Probes Recommended for Allelic Discrimination Assays

Applied Biosystems recommends the general use of TaqMan MGB probes for allelic discrimination assays, especially when conventional TaqMan probes exceed 30 nucleotides. The TaqMan MGB probes contain:

- A nonfluorescent quencher at the 3′ end - The SDS instruments can measure the reporter dye contributions more precisely because the quencher does not fluoresce.
- A minor groove binder at the 3′ end - The minor groove binder increases the melting temperature (T_m) of probes, allowing the use of shorter probes.

Consequently, the TaqMan MGB probes exhibit greater differences in T_m values between matched and mismatched probes, which provide more accurate allelic discrimination.

Advantages of TaqMan Chemistry

The advantages of the TaqMan chemistry are as follows:

- Specific hybridization between probe and target is required to generate fluorescent signal
- Probes can be labeled with different, distinguishable reporter dyes, which allows amplification of two distinct sequences in one reaction tube
- Post-PCR processing is eliminated, which reduces assay labor and material costs.

Disadvantage of TaqMan Chemistry

The primary disadvantage of the TaqMan chemistry is that the synthesis of different probes is required for different sequences.

SYBR® Green I Dye Chemistry

Background

Small molecules that bind to double-stranded DNA can be divided into two classes:

- Intercalators
- Minor-groove binders

Regardless of the binding method, there are two requirements for a DNA binding dye for real-time detection of PCR:

- Increased fluorescence when bound to double-stranded DNA
- No inhibition of PCR

Applied Biosystems has developed conditions that permit the use of the SYBR Green I dye in PCR without PCR inhibition and increased sensitivity of detection compared to ethidium bromide.

How the SYBR Green I Dye Chemistry Works

The SYBR Green I dye chemistry uses the SYBR Green I dye to detect polymerase chain reaction (PCR) products by binding to double-stranded DNA formed during PCR. Here's how it works:

Step Process

When SYBR Green I dye is added to a sample, it immediately binds to all double-stranded DNA present in the sample.

1. During the PCR, AmpliTaq Gold® DNA Polymerase amplifies the target sequence, which creates the PCR products, or "amplicons."
2. The SYBR Green I dye then binds to each new copy of double-stranded DNA.
3. As the PCR progresses, more amplicons are created. Since the SYBR Green I dye binds to all double-stranded DNA, the result is an increase in fluorescence intensity proportionate to the amount of PCR product produced.

Advantages of SYBR Green I Dye

The advantages of the SYBR Green I dye chemistry are as follows:

- It can be used to monitor the amplification of any double-stranded DNA sequence.
- No probe is required, which reduces assay setup and running costs.

Disadvantage of SYBR Green I Dye

The primary disadvantage of the SYBR Green I dye chemistry is that it may generate false positive signals; i.e., because the SYBR Green I dye binds to any double-stranded DNA, it can also bind to nonspecific double-stranded DNA sequences.

Additional Consideration

Another aspect of using DNA binding dyes is that multiple dyes bind to a single amplified molecule. This increases the sensitivity for detecting amplification products. A consequence of multiple dye binding is that the amount of signal is dependent on the mass of double-stranded DNA produced in the reaction. Thus, if the amplification efficiencies are the same, amplification of a longer product will generate more signal than a shorter one. This is in contrast to the use of a fluorogenic probe, in which a single fluorophore is released from quenching for each amplified molecule synthesized, regardless of its length.

About Quantitation Assays

What Is a Quantitation Assay?

A Quantitation Assay is a real-time PCR assay. It measures (quantitates) the amount of a nucleic acid target during each amplification cycle of the PCR. The target may be DNA, cDNA, or RNA. There are three types of Quantitation Assays discussed in this chemistry guide:

- DNA/cDNA quantitation
- RNA quantitation using one-step reverse transcription polymerase chain reaction (RT-PCR)
- RNA quantitation using two-step RT-PCR

Terms Used in Quantitation Analysis

Amplicon: A short segment of DNA generated by the PCR process
Amplification plot: The plot of fluorescence signal versus cycle number
Baseline: The initial cycles of PCR, in which there is little change in fluorescence signal
Ct (threshold cycle): The fractional cycle number at which the fluorescence passes the fixed threshold **NTC** (no template control) - A sample that does not contain template. It is used to verify amplification quality.
Nucleic acid target: (also called "target template") - DNA or RNA sequence that you wish to amplify
Passive **reference:** A dye that provides an internal reference to which the reporter dye signal can be

normalized during data analysis. Normalization is necessary to correct for forestallment fluctuations caused by changes in concentration or volume. A passive reference dye is included in all SDS PCR reagent kits.
Rn (normalized reporter): The fluorescence emission intensity of the reporter dye divided by the fluorescence emission intensity of the passive reference dye
Rn+: The Rn value of a reaction containing all components, including the template
Rn-: The Rn value of an un-reacted sample. The Rn-value can be obtained from:

- The early cycles of a real-time PCR run (those cycles prior to a detectable increase in fluorescence), OR
- A reaction that does not contain any template

ΔRn (delta Rn): The magnitude of the signal generated by the given set of PCR conditions. The ΔRn value is determined by the following formula: (Rn+) – (Rn-) **Standard** A sample of known concentration used to construct a standard curve. By running standards of varying concentrations, you create a standard curve from which you can extrapolate the quantity of an unknown sample.
Threshold: The average standard deviation of Rn for the early PCR cycles, multiplied by an adjustable factor. The threshold should be set in the region associated with an exponential growth of PCR product.
Unknown: A sample containing an unknown quantity of template. This is the sample whose quantity you want to determine.

How Real-Time PCR Quantitation Assays Work

In the initial cycles of PCR, there is little change in fluorescence signal. This defines the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated target. A fixed fluorescence threshold can be set above the baseline. The parameter C_T (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold.

Absolute vs. Relative Quantitation

Overview

When calculating the results of your quantitation assays, you can use either absolute or relative quantitation.

What is Absolute Quantitation?

The absolute quantitation assay is used to quantitate unknown samples by interpolating their quantity from a standard curve.

Example

Absolute quantitation might be used to correlate viral copy number with a disease state. It is of interest to the researcher to know the exact copy number of the target RNA in a given biological sample in order to monitor the progress of the disease.

Absolute quantitation can be performed with data from all of the SDS instruments, however, the absolute quantities of the standards must first be known by some independent means.

What is Relative Quantitation?

A relative quantitation assay is used to analyze changes in gene expression in a given sample relative to another reference sample (such as an untreated control sample).

Example
Relative quantitation might be used to measure gene expression in response to a chemical (drug). The level of gene expression of a particular gene of interest in a chemically treated sample would be compared relative to the level of gene expression an untreated sample.

Calculation Methods for Relative Quantitation
Relative quantitation can be performed with data from all of the SDS instruments. The calculation methods used for relative quantitation are:

- Standard curve method
- Comparative C_T method

Determining Which Method to Use
All methods can give equivalent results. When determining which method you want to use, note the following:

- Running the target and endogenous control amplifications in separate tubes and using the standard curve method of analysis requires the least amount of optimization and validation.
- To use the comparative C_T method, a validation experiment must be run to show that the efficiencies of the target and endogenous control amplifications are approximately equal. The advantage of using the comparative C_T method is that the need for a standard curve is eliminated. This increases throughput because wells no longer need to be used for the standard curve samples. It also eliminates the adverse effect of any dilution errors made in creating the standard curve samples.
- To amplify the target and endogenous control in the same tube, limiting primer concentrations must be identified and shown not to affect C_T values. By running the two reactions in the same tube, throughput is increased and the effects of pipetting errors are reduced.

Terms Used
The following terms are used in this discussion of absolute and relative quantitation:

Standard: A sample of known concentration used to construct a standard curve.
Reference: A passive or active signal used to normalize experimental results. Endogenous and exogenous controls are examples of active references. Active reference means the signal is generated as the result of PCR amplification. The active reference has its own set of primers and probe.
Endogenous control: This is an RNA or DNA that is present in each experimental sample as isolated. By using an endogenous control as an active reference, you can normalize quantitation of a messenger RNA (mRNA) target for differences in the amount of total RNA added to each reaction.
Exogenous control: This is a characterized RNA or DNA spiked into each sample at a known concentration. An exogenous active reference is usually an in vitro construct that can be used as an internal positive control (IPC) to distinguish true target negatives from PCR inhibition. An exogenous reference can also be used to normalize for differences in efficiency of sample extraction or complementary DNA (cDNA) synthesis by reverse transcriptase. Whether or not an active reference is used, it is important to use a passive reference containing the dye ROX in order to normalize for non-PCR-related fluctuations in fluorescence signal.
Normalized amount of target: A unitless number that can be used to compare the relative amount of target in different samples.
Calibrator: A sample used as the basis for comparative results.

Standard Curve Method for Relative Quantitation

Overview
It is easy to prepare standard curves for relative quantitation because quantity is expressed

relative to some basis sample, such as the calibrator. For all experimental samples, target quantity is determined from the standard curve and divided by the target quantity of the calibrator. Thus, the calibrator becomes the 1× sample, and all other quantities are expressed as an n-fold difference relative to the calibrator. As an example, in a study of drug effects on expression, the untreated control would be an appropriate calibrator.

Critical Guidelines

The guidelines below are critical for proper use of the standard curve method for relative quantitation:

- It is important that stock RNA or DNA be accurately diluted, but the units used to express this dilution are irrelevant. If two-fold dilutions of a total RNA preparation from a control cell line are used to construct a standard curve, the units could be the dilution values 1, 0.5, 0.25, 0.125, and so on. By using the same stock RNA or DNA to prepare standard curves for multiple plates, the relative quantities determined can be compared across the plates.

- It is possible to use a DNA standard curve for relative quantitation of RNA. Doing this requires the assumption that the reverse transcription efficiency of the target is the same in all samples, but the exact value of this efficiency need not be known.

- For quantitation normalized to an endogenous control, standard curves are prepared for both the target and the endogenous reference. For each experimental sample, the amount of target and endogenous reference is determined from the appropriate standard curve. Then, the target amount is divided by the endogenous reference amount to obtain a normalized target value. Again, one of the experimental samples is the calibrator, or 1× sample. Each of the normalized target values is divided by the calibrator normalized target value to generate the relative expression levels.

Endogenous Control

Amplification of an endogenous control may be performed to standardize the amount of sample RNA or DNA added to a reaction. For the quantitation of gene expression, researchers have used ß-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal RNA (rRNA), or other RNAs as an endogenous control.

Standards

Because the sample quantity is divided by the calibrator quantity, the unit from the standard curve drops out. Thus, all that is required of the standards is that their relative dilutions be known. For relative quantitation, this means any stock RNA or DNA containing the appropriate target can be used to prepare standards.

Comparative C_T method for Relative Quantitation

The comparative C_T method is simlar to that standard curve method, except it uses the arithmetic formula, $2^{-\Delta\Delta C_T}$ to achieve the same result for relative quantitation.

Arithmetic Formulas:

For the comparative CT method to be valid, the efficiency of the target amplification (your gene of interest) and the efficiency of the reference amplification (your endogenous control) must be

approximately equal.

For more information on using the comparative CT method for relative quantitation, please refer to User Bulletin #2: Relative Quantitation of Gene Expression (PN 4303859).

Standard Curve Method for Absolute Quantitation

Overview

The standard curve method for absolute quantitation is similar to the standard curve method for relative quantitation, except the absolute quantities of the standards must first be known by some independent means.

Critical Guidelines

The guidelines below are critical for proper use of the standard curve method for absolute quantitation:

- It is important that the DNA or RNA be a single, pure species. For example, plasmid DNA prepared from E. coli often is contaminated with RNA, which increases the A260 measurement and inflates the copy number determined for the plasmid.
- Accurate pipetting is required because the standards must be diluted over several orders of magnitude. Plasmid DNA or in vitro transcribed RNA must be concentrated in order to measure an accurate A260 value. This concentrated DNA or RNA must then be diluted 106–1012 -fold to be at a concentration similar to the target in biological samples.
- The stability of the diluted standards must be considered, especially for RNA. Divide diluted standards into small aliquots, store at –80 °C, and thaw only once before use.

It is generally not possible to use DNA as a standard for absolute quantitation of RNA because there is no control for the efficiency of the reverse transcription step.

Standards

The absolute quantities of the standards must first be known by some independent means. Plasmid DNA and in vitro transcribed RNA are commonly used to prepare absolute standards. Concentration is measured by A_{260} and converted to the number of copies using the molecular weight of the DNA or RNA.

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The SYBR® Green dye is sold pursuant to a limited license from Molecular Probes, Inc.

Applied Biosystems is a registered trademark and AB (Design) and TAMRA are trademarks of Applera Corporation or its subsidiaries in the US and certain other countries.

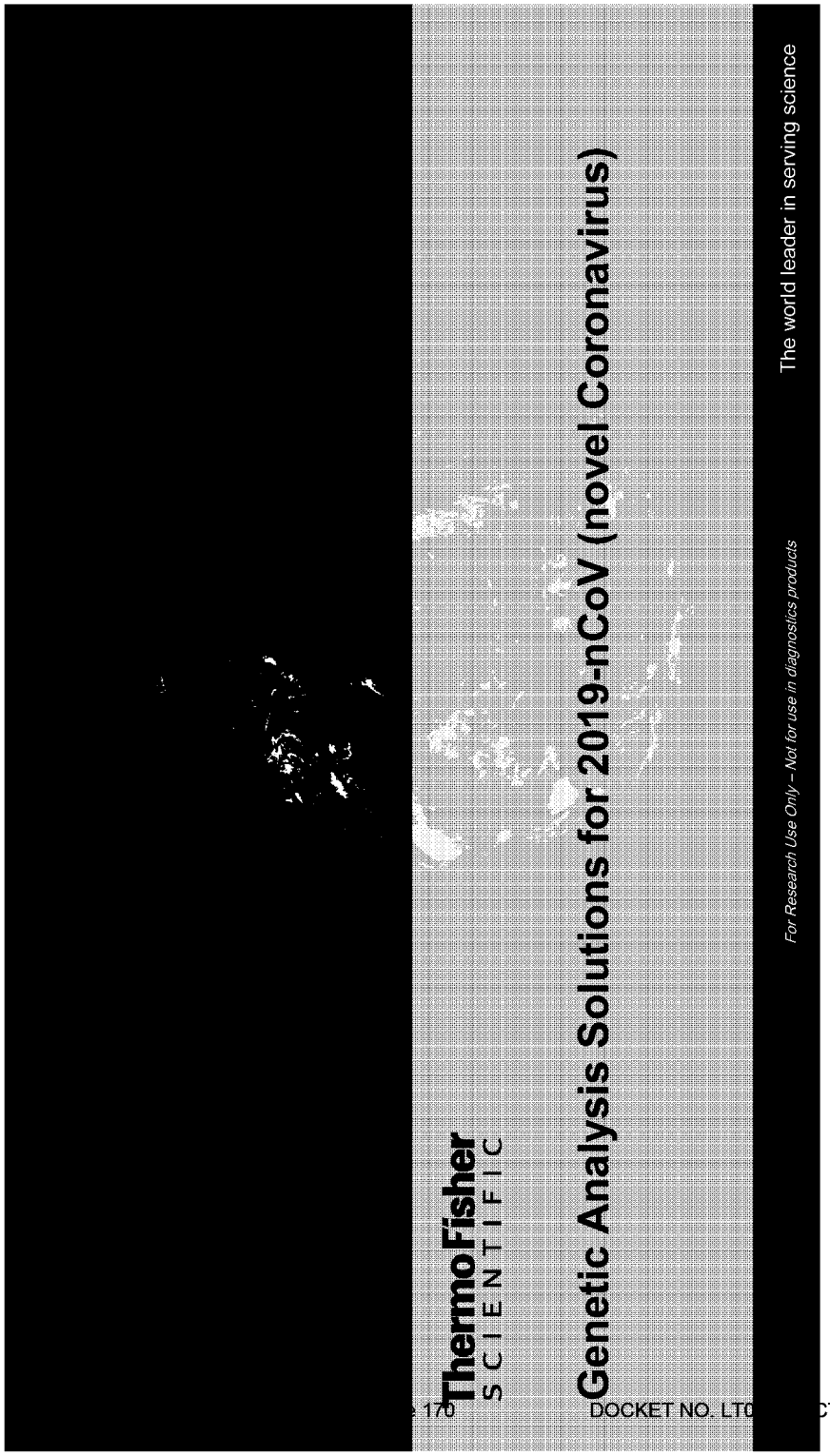
AmpliTaq Gold and TaqMan are registered trademarks of Roche Molecular Systems, Inc.

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117GU15-01

Part Number 4371089 Revision A

APPENDIX 5


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Genetic Analysis Solutions for 2019-nCoV (novel Coronavirus)
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Coronavirus Family – Global Impact in Humans

- Coronaviruses are named for the crown-like spikes on their surface. There are four main sub-groupings of coronaviruses, known as alpha, beta, gamma, and delta. Human coronaviruses were first identified in the mid-1960s. The seven coronaviruses that can infect humans are:
- Common human coronaviruses
  - 229E (alpha coronavirus)
  - NL63 (alpha coronavirus)
  - OC43 (beta coronavirus)
  - HKU1 (beta coronavirus)

People around the world commonly get infected with human coronaviruses 229E, NL63, OC43, and HKU1

- Coronaviruses can infect animals and evolve and undergo zoonosis to new human coronavirus. Coronaviruses that have crossed over to humans are:
  - MERS-CoV (the beta coronavirus that causes Middle East Respiratory Syndrome, or MERS)
  - SARS-CoV (the beta coronavirus that causes severe acute respiratory syndrome, or SARS)
  - 2019 Novel Coronavirus (2019-nCoV)

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TaqMan qPCR Assays for Coronaviruses Family

Product Type	SKU	Assay ID	Assay Name	Price
TaqMan™ Gene Expression Assay	4331182	Vi06439671_s1	Human Coronavirus 229E	List
TaqMan™ Gene Expression Assay	4331182	Vi06439674_s1	Human Coronavirus HKU1	List
TaqMan™ Gene Expression Assay	4331182	Vi06439673_s1	Human Coronavirus NL63	List
TaqMan™ Gene Expression Assay	4331182	Vi06439646_s1	Human Coronavirus OC43	List
TaqMan™ Gene Expression Assay	4331182	Vi06439644_s1	Middle East Respiratory Syndrome coronavirus (MERS)	List
TaqMan™ Gene Expression Assay	4331182	Vi06439634_s1	Severe Acute Respiratory Syndrome coronavirus (SARS)	List

Above assays are orderable on the web for customers
Primary use case for a customer is to differentiate samples with known coronaviruses
Available in small, medium and large sizes (similar to other inventoried TaqMan Gene Expression assays)

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Generation 1: 2019-nCoV Assay Kit and Control

SKU	SKU Name	Unit	Product Description	Unit Size	Status
A47532	TaqMan 2019nCoV Assay Kit v1	1 wet tube	2019nCoV Assay (Gene Orf-1ab) – FAM, 20X	50 reactions (75 ul)	Feb 5th Launch
		1 wet tube	2019nCoV Assay (Gene S protein) – FAM, 20X	50 reactions (75 ul)	
		1 wet tube	2019nCoV Assay (Gene N protein) – FAM, 20X	50 reactions (75 ul)	
		1 wet tube	RNase P – VIC, 20X	150 reactions (225 ul)	
A47533	TaqMan 2019nCoV Control Kit v1	1 wet tube	2019nCoV DNA Control (covering Gene Orf-1ab, Gene S protein and Gene N protein, RNase P)	50 ul (Conc 1X10^4 copies/uL)	Feb 5th Launch

- **Use Case:** Evaluation of respiratory samples in laboratory settings
- Kit assays target all 52 currently available complete genomes at GISAID and do not target any of the 2116 complete genomes of other coronaviruses currently available at NCBI
- Larger units of assays and controls available on special order
- Master Mix (Recommendation): TaqPath™ 1-Step RT-qPCR Master Mix, CG (Cat. No. A15299)

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Master Mixes recommended for 2019 nCoV assay detection

- **TaqPath 1-Step RT-qPCR Master Mix, CG (PN - A15299)**
  - All the advantages of TaqMan Fast Virus 1-step Master Mix
  - Includes UNG and dUTP for carryover contamination control
  - Enhanced quality control measures for even better lot to lot consistency
  - For use primarily in the US, as written into key protocols by the US CDC
  - *General purpose reagent*
- **TaqMan Fast Virus 1-step Master Mix (PN - 4444434, 4444432, 4444436)**
  - Highly concentrated (4X) master mix – ability to add more sample for improved sensitivity
  - Exhibits inhibitor tolerance, especially those found in difficult sample types, provides greater accuracy
  - One-tube master mix for improved efficiency and ease of use
  - Optimized for multiplexing (up to 3 targets) reactions and with exogenous/endogenous internal controls
  - Fast cycling – provides results in ~60 min, half the time of standard cycling (also works with standard cycling)
  - TaqPath 1-step MMx is suggested for EMEA, China, and APJ due to faster short term availability
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The above two Master Mixes are preferably recommended for 2019 nCoV assay detection

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Generation 2: TaqMan™ Array Respiratory Tract Microbiota Card with 2019nCoV

Use Case: Syndromic Evaluation & Epidemiological Studies

SKU	Protocol Step	SKU Name	Unit	Format	Price
4398986 (Generic)	TAC	TaqMan Array Card, RTM w 2019nCoV	1 Card	Format 48	$500
4441856 (Generic)	PreAmp	TaqMan PreAmp Pool, RTM w 2019nCoV	1 ml		$350
A15299	PreAmp	TaqPath™ 1-Step RT-qPCR Master Mix, CG	5 x 1 ml		LIST
A44359	qPCR	TaqMan® Fast Advanced Master Mix, No UNG	1ml		LIST
A44360	qPCR	TaqMan® Fast Advanced Master Mix, No UNG	5ml		LIST
A30220 (Generic)	Control	Custom Control, RTM w 2019nCoV	250 ul	10^5 cp/ul	$750

The **RTM** (Respiratory Tract Microbiota) Taqman Array Card, V6 is a customized version of the launched product: TaqMan™ Array Respiratory Tract Microbiota Comprehensive Card (SKU: **A41238**)
Link: https://www.thermofisher.com/order/catalog/product/A41238

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Generation 1 CoV Detection Product Performance:
TaqMan 2019nCoV Assay Kit v1
TaqMan 2019nCoV Control Kit v1
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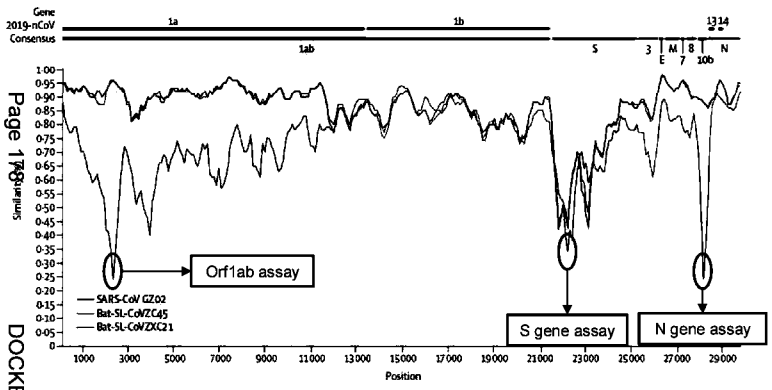
2019 Novel Coronavirus nCoV Assay Designs
Assay Offering
Designed assays targeting:
• ORF1ab
• N protein
• S protein (unique offering)
Coverage
Global Initiative on Sharing All Influenza Data (GISAID)
Target 100% of all 52* currently available complete genomes for 2019 nCoV
Specificity
National Center for Biotechnology Information (NCBI)
Specific for 2019 nCoV against:
• 2116 other complete genomes of coronaviruses in NCBI
Highly effective designs for targeting 2019 Novel Coronavirus
*As of January 31, 2020. Genomes are being frequently updated to GISAID because of the extreme interest in this emergence. Percent coverage is being updated by Thermo Fisher Scientific as this information becomes available
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Specific Assays Target Regions of Reduced Homology

A

	Strain	Complete genome (%)	Gene region (%)											
			1ab	1a	1b	S	3	E	M	7	8	10b	N	13	14
Nucleotide sequences	Bat-SL-CoVZC45	87·6	88·9	90·7	86·0	75·2	87·8	98·7	93·4	95·2	88·8	88·5	91·1	89·1	96·7
	Bat-SL-CoVZXC21	87·5	88·7	90·3	86·1	74·7	88·9	98·7	93·4	95·2	89·1	88·5	91·2	89·5	96·7
	SARS-CoVGZ02	79·0	79·5	75·4	86·3	72·7	75·6	93·5	85·1	74·5	82·1	..	88·1	..	..
Amino acid sequences	Bat-SL-CoVZC45	..	95·6	95·6	95·8	80·2	90·9	100·0	98·6	93·4	87·6	94·2	94·3	73·2	92·9
	Bat-SL-CoVZXC21	..	95·2	95·1	95·5	79·6	92·0	100·0	98·6	93·4	88·4	94·2	94·3	73·2	92·9
	SARS-CoVGZ02	..	86·2	80·5	95·6	76·2	73·1	94·7	90·1	68·9	85·2	..	90·3	..	..

B



Sequence identity for 2019-nCoV compared with SARS-CoV GZ02 (accession number AY390556) and the bat SARS-like coronaviruses bat-SL-CoVZC45 (MG772933) and bat-SL-CoVZXC21 (MG772934).

The three genes targeted in our assays are specifically designed to regions that are strain specific.

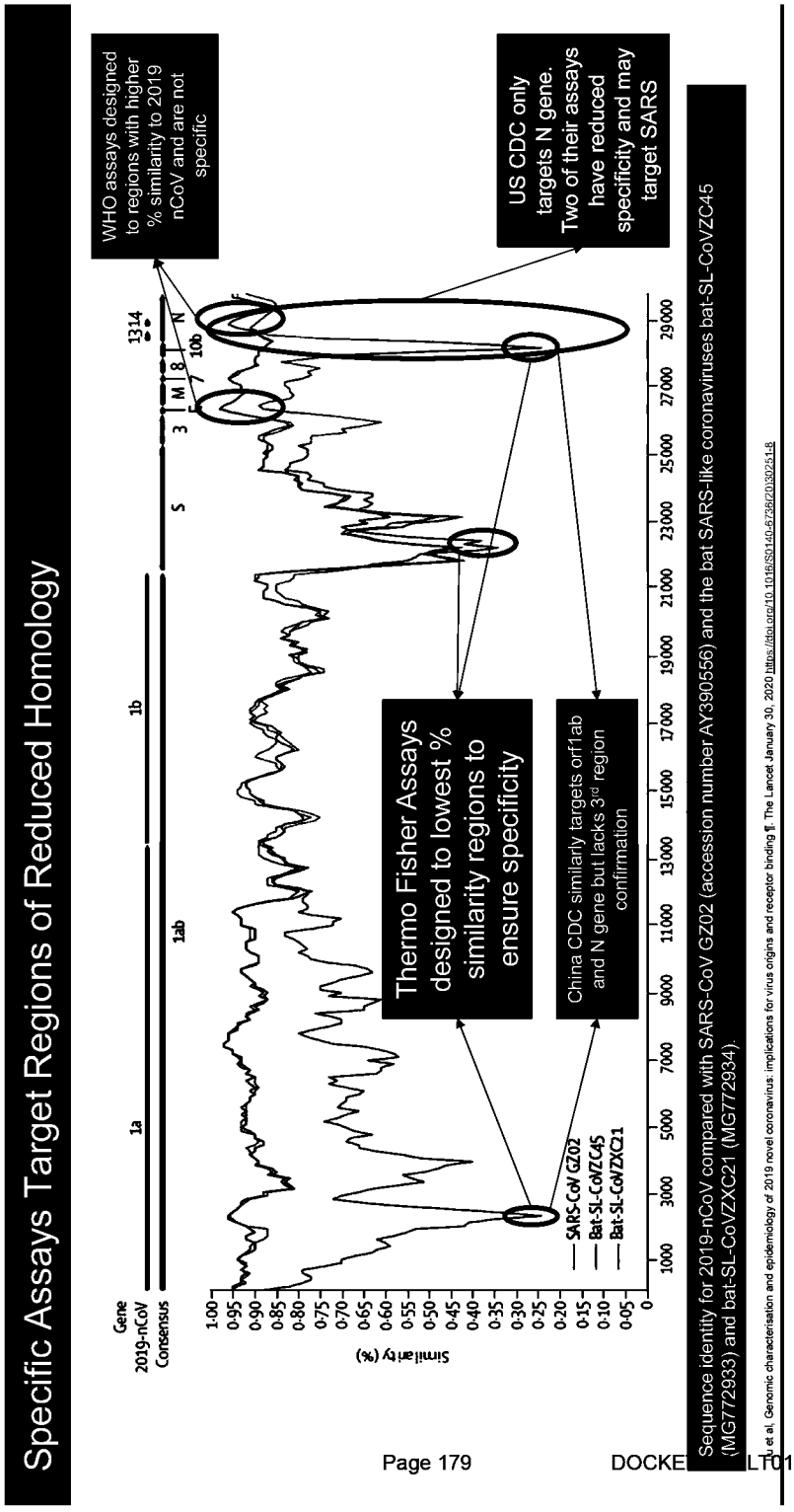
- Orf1ab: 1000 – 3000 region
- S gene: 21500 – 23000 region
- N gene: 28000 – 29500 region

When 2019 nCoV is mapped against SARS-CoV, it shows significantly reduced % similarity at three troughs.*

These evident troughs are ideal for identifying 2019 nCoV and are what our assays target.

Competitor RUO assays available in the market also target similarly N and orf1ab, however targeting S gene is unique to Thermo Fisher and confirmed as an ideal candidate in the Lancet publication by Lu et al.

* Lu et al.. Genomic characterization and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding ¶. The Lancet January 30, 2020 https://doi.org/10.1016/S0140-6736(20)30251-8


Specific Assays Target Regions of Reduced Homology
WHO assays designed to regions with higher % similarity to 2019 nCoV and are not specific
US CDC only targets N gene. Two of their assays have reduced specificity and may target SARS
Thermo Fisher Assays designed to lowest % similarity regions to ensure specificity
China CDC similarity targets orf1ab and N gene but lacks 3rd region confirmation
Gene
2019-nCoV
Consensus
1a
1b
1ab
S
3
M
8
10b
N
1314
Similarity (%)
SARS-CoV GZ02
Bat-SL-CoVZC45
Bat-SL-CoVZXC21
Sequence identity for 2019-nCoV compared with SARS-CoV GZ02 (accession number AY390556) and the bat SARS-like coronaviruses bat-SL-CoVZC45 (MG772933) and bat-SL-CoVZXC21 (MG772934).
Lu et al. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding ¶. The Lancet January 30, 2020 https://doi.org/10.1016/S0140-6736(20)30251-8
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How our Assays Match Up: In Silico Evaluation

Design Criteria	Thermo Fisher	China CDC	United States CDC	WHO
Target Regions	• Orf1ab* • N Protein* • S Protein*	• Orf1ab (3 assays) • N Protein (Both targets positive for confirmation)	• N Protein (3 assays)	• E Protein (screening) • N Protein(confirmatory) • RdRp-SARS/Wuhan (confirmatory)
Coverage	100% coverage of published genomes	100% coverage of published genomes	100% coverage of published genomes	100% coverage of published genomes
Specificity	All assays are specific	All assays are specific	Two assays (N1 and N3) might generate a signal if SARS or Bat-SARS-CoV are present in higher concentrations.	E and N protein assays map perfectly to hundreds of SARS and other CoV strains. RdRp SARS/Wuhan assay was designed to detect both SARS and 2019nCoV, so it's not specific by design.
Control Assays	RNaseP	No recommendation	RNaseP	No recommendation

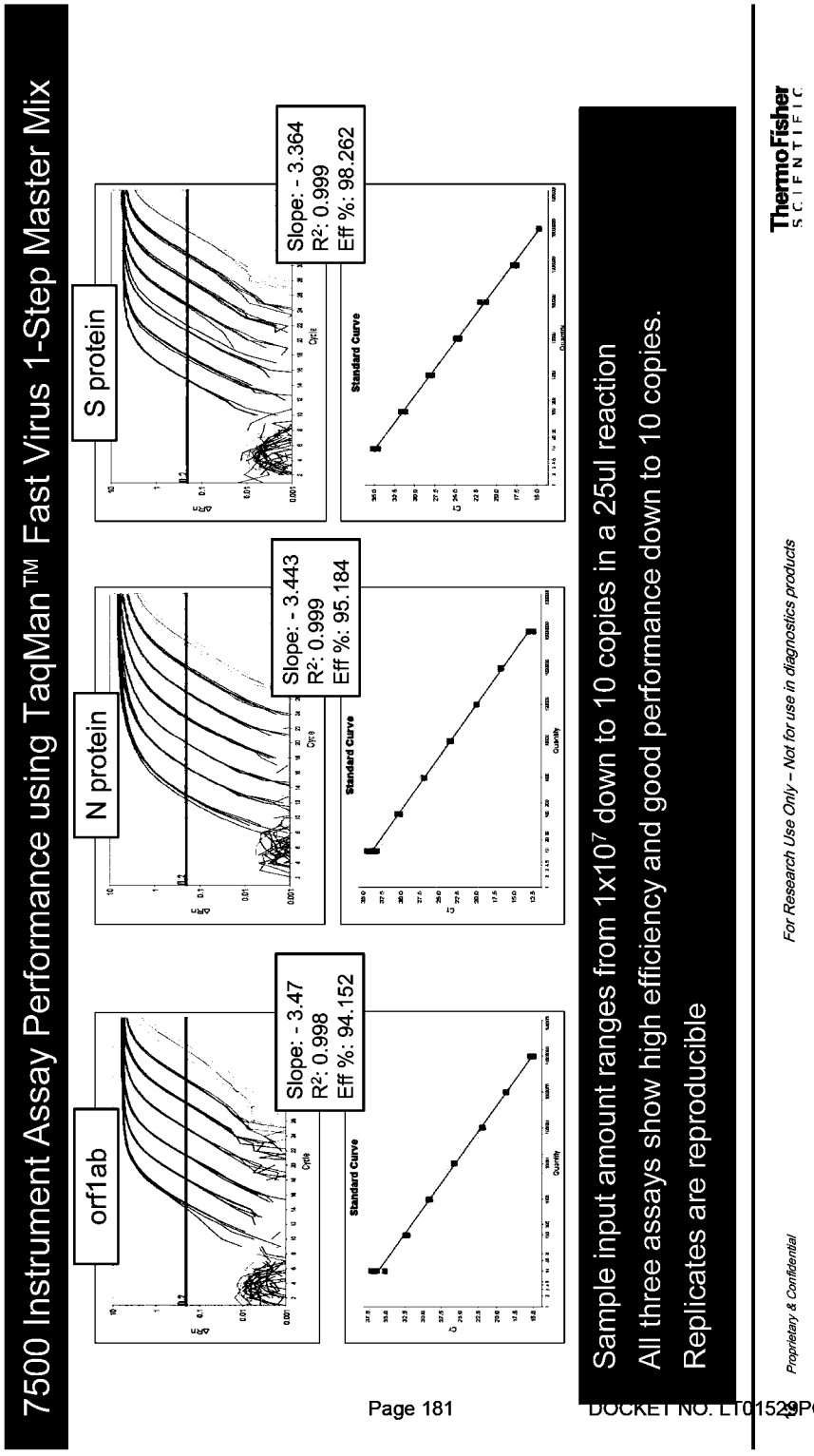
* All of the TaqMan Assays targeting Orf1ab, N Protein and S Protein are unique designs and do not overlap with any designs from US CDC, China CDC or WHO protocols

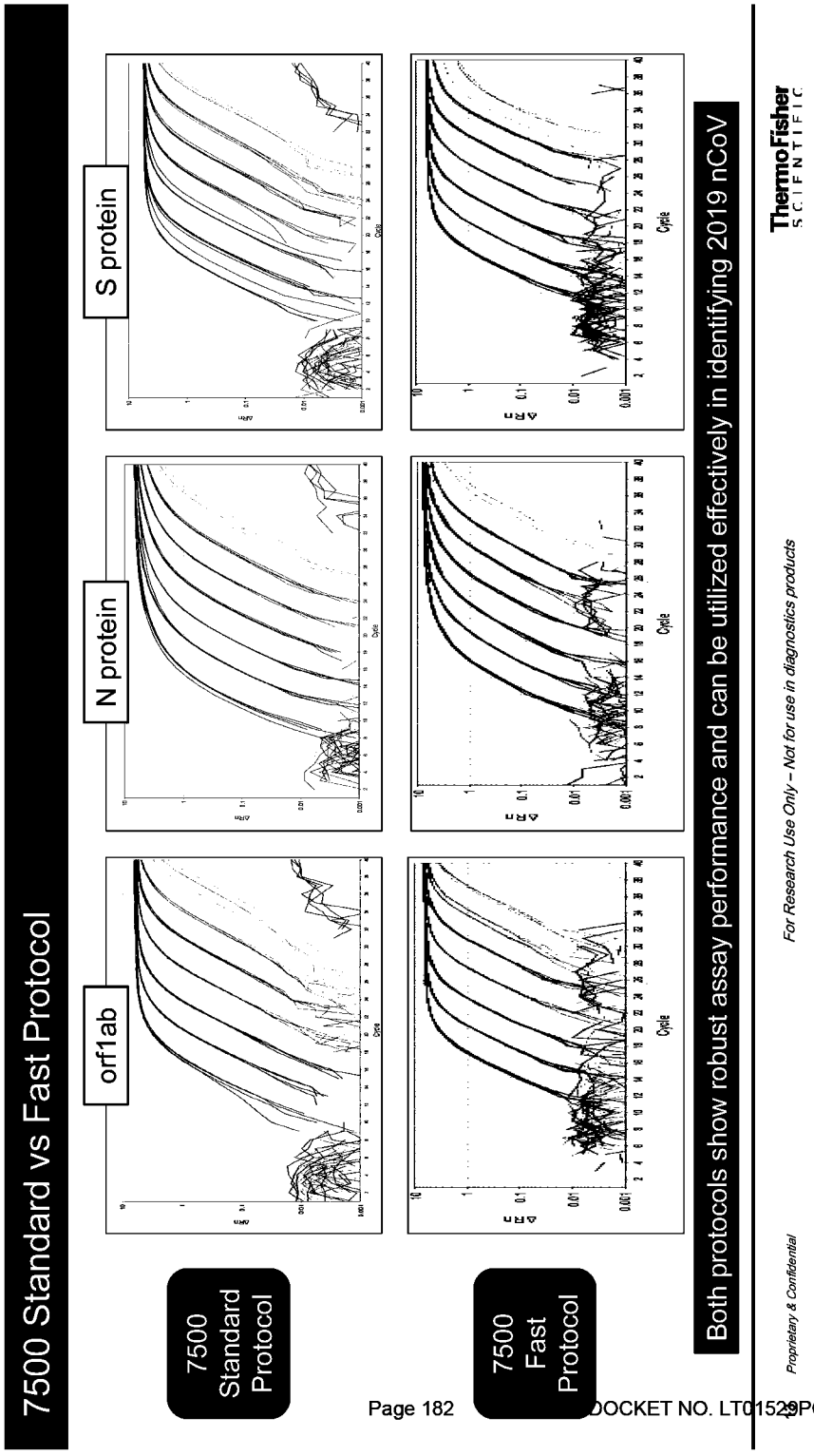
All statements are based on in silico mapping to 52 complete genomes. The mapping was done using our standard mapping tool zpcr3p. All our assays' matches to 52 genomes were perfect – no mismatches.

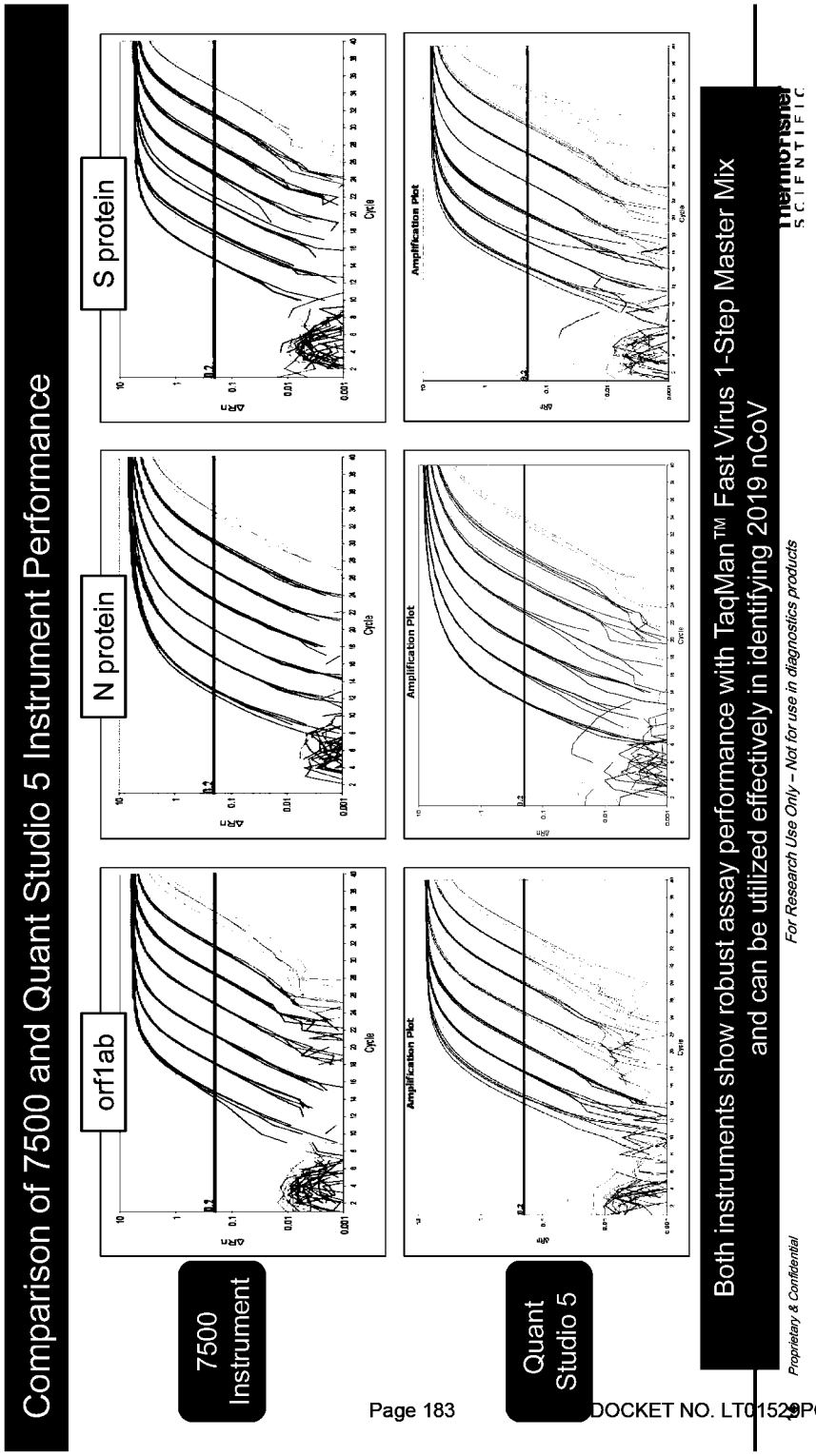
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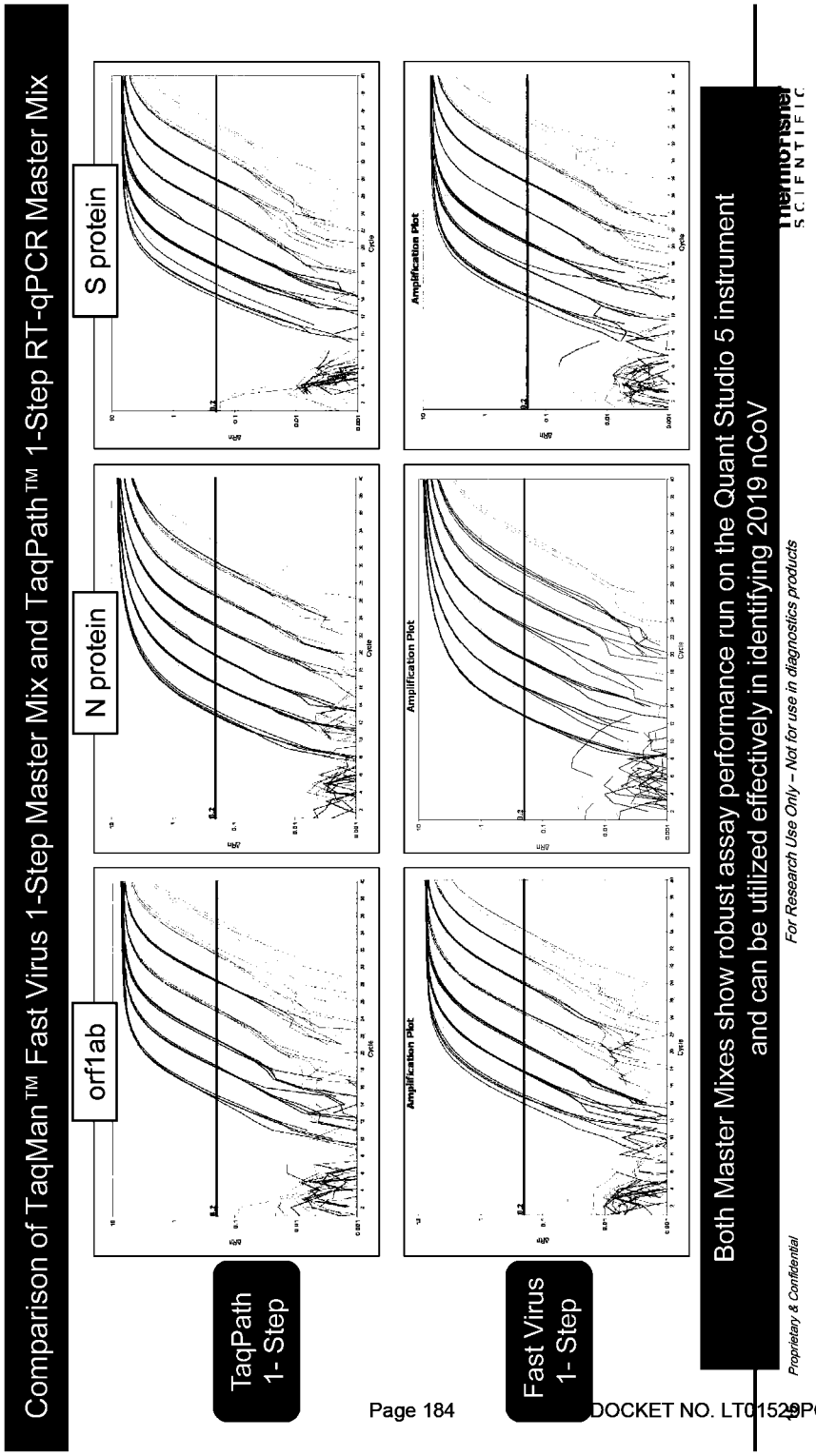
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7500 Instrument Assay Performance using TaqMan™ Fast Virus 1-Step Master Mix
orf1ab
Slope: - 3.47
R²: 0.998
Eff %: 94.152
N protein
Slope: - 3.443
R²: 0.999
Eff %: 95.184
S protein
Slope: - 3.364
R²: 0.999
Eff %: 98.262
Standard Curve
Sample input amount ranges from 1x10⁷ down to 10 copies in a 25ul reaction
All three assays show high efficiency and good performance down to 10 copies.
Replicates are reproducible
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7500 Standard vs Fast Protocol
orf1ab
N protein
S protein
7500 Standard Protocol
7500 Fast Protocol
Cycle
ΔRn
Both protocols show robust assay performance and can be utilized effectively in identifying 2019 nCoV
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Comparison of 7500 and Quant Studio 5 Instrument Performance
orf1ab
N protein
S protein
7500 Instrument
Quant Studio 5
Amplification Plot
Cycle
Both instruments show robust assay performance with TaqMan™ Fast Virus 1-Step Master Mix and can be utilized effectively in identifying 2019 nCoV
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Comparison of TaqMan™ Fast Virus 1-Step Master Mix and TaqPath™ 1-Step RT-qPCR Master Mix
orf1ab
N protein
S protein
TaqPath 1- Step
Fast Virus 1- Step
Amplification Plot
Both Master Mixes show robust assay performance run on the Quant Studio 5 instrument and can be utilized effectively in identifying 2019 nCoV
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Assay Performance Comparison

Target	Instrument	MasterMix	Slope	R^2	Efficiency
Orf1ab	ABI 7500	TaqMan® Fast Virus 1-Step Master Mix (Standard)	-3.47	0.998	94.152 %
	QuantStudio™ 5	TaqMan® Fast Virus 1-Step Master Mix (Standard)	-3.321	0.998	100.022 %
		TaqPath™ 1-Step RT-qPCR Master Mix, CG	-3.385	0.999	97.418 %
N protein	ABI 7500	TaqMan® Fast Virus 1-Step Master Mix (Standard)	-3.443	0.999	95.184 %
	QuantStudio™ 5	TaqMan® Fast Virus 1-Step Master Mix (Standard)	-3.417	0.999	96.184 %
		TaqPath™ 1-Step RT-qPCR Master Mix, CG	-3.324	0.995	99.909 %
S protein	ABI 7500	TaqMan® Fast Virus 1-Step Master Mix (Standard)	-3.364	0.999	98.262 %
	QuantStudio™ 5	TaqMan® Fast Virus 1-Step Master Mix (Standard)	-3.408	0.997	96.51 %
		TaqPath™ 1-Step RT-qPCR Master Mix, CG	-3.319	0.998	100.114 %

Assays show good overall performance regardless of instrument and master mix used

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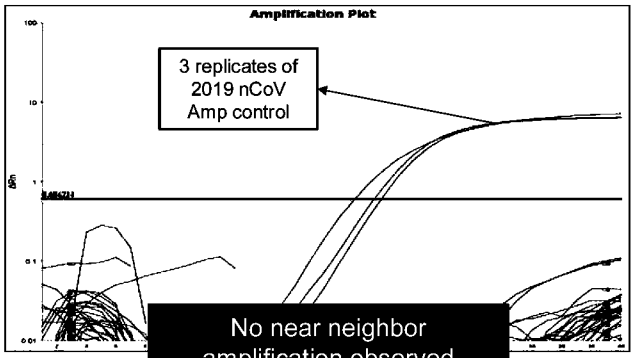
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Specificity Data

Sample Tested	Sample Information*	Orf1ab	N Protein	S Protein
Coronavirus OC43	0810024CF	No Amp	No Amp	No Amp
Coronavirus NL63	0810228CF	No Amp	No Amp	No Amp
Coronavirus 229E	0810229CF	No Amp	No Amp	No Amp
Coronavirus HKU1-1	Clinical Isolate	No Amp	No Amp	No Amp
Coronavirus HKU1-2	Clinical Isolate	No Amp	No Amp	No Amp
Flu A H3	0810252CF	No Amp	No Amp	No Amp
Flu A H1N1	0810244CF	No Amp	No Amp	No Amp
Flu B	0810255CF	No Amp	No Amp	No Amp
RSV A	0810040ACF	No Amp	No Amp	No Amp
RSV B	0810040CF	No Amp	No Amp	No Amp
Rhinovirus	0810014CF	No Amp	No Amp	No Amp
PIP 1	0810015CF	No Amp	No Amp	No Amp
PIV 2	0810016CF	No Amp	No Amp	No Amp
PIV 3	0810060CF	No Amp	No Amp	No Amp
PIV 4	0810012CFN	No Amp	No Amp	No Amp
Bordetella pertussis	0801460	No Amp	No Amp	No Amp
NTC		No Amp	No Amp	No Amp

*Samples with ID's listed were organism controls acquired from ZeptoMetrix Corporation

- The assays were tested with related organisms to showcase specificity of design.
- It was also tested with the amplification control to confirm adequate assay performance.
- No amplification was seen from any near neighbor pathogens, confirming the *in silico* analysis results.
- These assays are specific to 2019-nCoV.



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NTC Performance
Amplification Plot
Amplification Plot
Amplification Plot
Cycle
Cycle
Cycle
Target
Orf1ab
S protein
N protein
CT Undetermined
(12 replicates)
100%
12/12
100%
12/12
100%
12/12
NTC replicates are clean and do not yield any Ct values for the 2019 nCoV assays. They are all listed as undetermined
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2019 nCoV Synthetic Control Information
orf1ab
N protein
S protein
RNase P
To test performance of assays targeting 2019 nCoV, we provide a synthetic plasmid control.
The control includes the assay target insert for all three targets:
Gene orf1ab
Gene S protein
Gene N protein
It also incorporates control target:
RNaseP
The control is provided at 1x10^4 copies/ul (Digitally quantified)
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Experiment Setup using TaqPath™ 1-Step RT-qPCR Master Mix, CG

PCR Reaction Mix Component	Volume per well (25 ul reaction)
	Standard 96-well 0.2 ml Plates
TaqPath™ 1-Step RT-qPCR Master Mix, CG (4X)	6.25 µL
2019 nCoV TaqMan Assay (20X) (FAM)	1.25 µL
TaqMan® RNase P Assay, VIC™ dye/QSY™ assay (20X)	1.25 µL
Nuclease-free water	11.25 µL
Purified Sample	5.00 µL
Final Reaction Volume	25.00 µL

Example Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A			ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P		S Protein RNase P	S Protein RNase P			N Protein RNase P	N Protein RNase P
B	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
C	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
D	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
E	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
F	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
G	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
H	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P

NTC | Positive Control | Test sample

TaqPath™ 1-Step RT-qPCR Master Mix, CG

Catalog Numbers A15299, A15300

Publication Number MAN0007959

Step	Stage	No. of cycles	Temperature	Time
UNG incubation	1	1	25°C	2 minutes
Reverse transcription*	2	1	50°C†	15 minutes
Polymerase activation‡	3	1	95°C‡	2 minutes
Amplification	4	40	95°C	15 seconds
			60°C	60 seconds

* Reverse transcription works best between 48°C and 55°C.
† Required for RT inactivation, initial denaturation, and to activate the DNA polymerase.

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Experiment Setup using TaqMan® Fast Virus 1-Step Master Mix

PCR Reaction Mix Component	Volume per well (25 ul reaction)
	Standard 96–well 0.2 ml Plates
TaqMan Fast Virus 1-Step RT-qPCR Master Mix, CG (4X)	6.25 µL
2019 nCoV TaqMan® Assay (20X) (FAM)	1.25 µL
TaqMan® RNase P Assay, VIC™ dye/QSY™ assay (20X)	1.25 µL
Nuclease-free water	11.25 µL
Purified Sample	5.00 µL
Final Reaction Volume	25.00 µL

TaqMan® Fast Virus 1-Step Master Mix

Catalog Numbers 4444432, 4444434, 4444436

Publication Part Number 4453800

Step	Stage	No. of cycles	Temperature	Time
Reverse transcription	1	1	50°C†	5 minutes
RT inactivation/initial denaturation	2	1	95°C	20 seconds
Amplification	3	40	95°C	15 seconds
			60°C	60 seconds

† Reverse transcription works best between 48°C and 55°C.

Example Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	[redacted]	[redacted]	ORF1ab RNase P	ORF1ab RNase P	[redacted]	[redacted]	S Protein RNase P	S Protein RNase P	[redacted]	[redacted]	N Protein RNase P	N Protein RNase P
B	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
C	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
D	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
E	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
F	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
G	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
H	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P

NTC | Positive Control | Test sample

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Software and Analysis Recommendations

Instrument	Software version	Analysis Settings
Quant Studio 5	QuantStudio Design & Analysis Software v1.5.1	Autobaseline Manual Threshold = 0.2 for all assays
7500 Real-Time PCR System	7500 Software v2.3	Autobaseline Manual Threshold = 0.2 for all assays

Ct Value	Result
Ct < 37 for at least two targets	Positive
37 ≤ Ct < 40 or Undetermined for 2 or more targets	Repeat the test.
Crt = Undetermined for all 3 assays	Negative

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Product Availability & Support

SKU	Product	Availability in US
A47532	2019nCoV Assay Kit v1 (Combo with three CoV assays & Rnase P control)	Feb 5th, 2020
A47532	2019nCoV Control Kit v1 (covering three CoV assays)	Feb 5th, 2020
	Kit Protocol	Feb 5th, 2020
4398986	Taqman Array Card, RTM v6 w 2019nCoV	Feb 20th, 2020
A30220	RTM Control v2 with 2019nCoV	Feb 20th, 2020
	TAC Protocol (Generic RTM TAC Protocol available)	Feb 28th, 2020

Mail Box for orders / inquiries:

- 2019nCoV Support: coronavirus@thermofisher.com

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Appendix
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DOCKET NO. LT0
CT

e 194
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Genetic Analysis Solutions for 2019-nCoV (novel Coronavirus)
DOCKET NO. LTC CT
February 18th, 2020
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Coronavirus Family – Global Impact in Humans

- Coronaviruses are named for the crown-like spikes on their surface. There are four main sub-groupings of coronaviruses, known as alpha, beta, gamma, and delta. Human coronaviruses were first identified in the mid-1960s. The seven coronaviruses that can infect humans are:
- Common human coronaviruses
  - 229E (alpha coronavirus)
  - NL63 (alpha coronavirus)
  - OC43 (beta coronavirus)
  - HKU1 (beta coronavirus)

People around the world commonly get infected with human coronaviruses 229E, NL63, OC43, and HKU1

- Coronaviruses can infect animals and evolve and undergo zoonosis to new human coronavirus. Coronaviruses that have crossed over to humans are:
  - MERS-CoV (the beta coronavirus that causes Middle East Respiratory Syndrome, or MERS)
  - SARS-CoV (the beta coronavirus that causes severe acute respiratory syndrome, or SARS)
  - 2019 Novel Coronavirus (2019-nCoV)

OTHER RECENT EPIDEMICS NEW STRAIN

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Applied Biosystems TaqMan qPCR Assays for Coronaviruses Family

Product type	SKU	Assay ID	Assay name
TaqMan Gene Expression Assay	4331182	Vi06439671_s1	Human Coronavirus 229E
TaqMan Gene Expression Assay	4331182	Vi06439674_s1	Human Coronavirus HKU1
TaqMan Gene Expression Assay	4331182	Vi06439673_s1	Human Coronavirus NL63
TaqMan Gene Expression Assay	4331182	Vi06439646_s1	Human Coronavirus OC43
TaqMan Gene Expression Assay	4331182	Vi06439644_s1	Middle East Respiratory Syndrome coronavirus (MERS)
TaqMan Gene Expression Assay	4331182	Vi06439634_s1	Severe Acute Respiratory Syndrome coronavirus (SARS)

Above assays are orderable on the web for customers
Primary use case for a customer is to differentiate samples with known coronaviruses
Available in small, medium and large sizes (similar to other inventoried Applied Biosystems™ TaqMan® Gene Expression Assays)

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Generation 1: 2019-nCoV Assay Kit and Control

SKU	SKU name	Unit	Product description	Unit size	Status
A47532	TaqMan 2019nCoV Assay Kit v1	1 wet tube	2019nCoV Assay (Gene Orf-1ab) – FAM, 20X	50 reactions (75 ul)	Available
		1 wet tube	2019nCoV Assay (Gene S protein) – FAM, 20X	50 reactions (75 ul)	
		1 wet tube	2019nCoV Assay (Gene **N** protein) – FAM, 20X	50 reactions (75 ul)	
		1 wet tube	RNase P – VIC, 20X	150 reactions (225 ul)	
A47533	TaqMan 2019nCoV Control Kit v1	1 wet tube	2019nCoV DNA Control (covering Gene Orf-1ab, Gene S protein and Gene N protein, RNase P)	50 ul (Conc 1X10^4 copies/uL)	Available

Use Case: Evaluation of respiratory samples in laboratory settings

- Kit assays target all 71 currently available complete genomes at GISAID and do not target any of the 2116 complete genomes of other coronaviruses currently available at NCBI

Larger units of assays and controls available on special order

Applied Biosystems™ TaqMan® 2019nCoV Assay Kit v1 & TaqMan® 2019nCoV Control Kit v1 are manufactured at different physical locations

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2019 nCoV Synthetic Control Information
orf1ab
N protein
S protein
RNase P
Xeno
To test performance of assays targeting 2019 nCoV, we provide a synthetic plasmid control.
The control includes the assay target insert for all three targets:
• Gene orf1ab
• Gene S protein
• Gene N protein
It also incorporates control target:
• RNaseP
• Xeno (included, if needed as a contamination marker)
The control is provided at 1x10⁴ copies/ul (Digitally quantified)
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Master Mixes Recommended for 2019 nCoV Assay Detection

- **Applied Biosystems™ TaqPath™ 1-Step RT-qPCR Master Mix, CG (PN - A15299)**
  - All the advantages of Applied Biosystems™ TaqMan® Fast Virus 1-step Master Mix
  - Includes UNG and dUTP for carryover contamination control
  - Enhanced quality control measures for even better lot to lot consistency
  - For use primarily in the US, as written into key protocols by the US CDC
  - *General purpose reagent*
- **Applied Biosystems™ TaqMan® Fast Virus 1-step Master Mix (PN - 4444434, 4444432, 4444436)**
  - Highly concentrated (4X) master mix – ability to add more sample for improved sensitivity
  - Exhibits inhibitor tolerance, especially those found in difficult sample types, provides greater accuracy
  - One-tube master mix for improved efficiency and ease of use
  - Optimized for multiplexing (up to 3 targets) reactions and with exogenous/endogenous internal controls
  - Fast cycling – provides results in ~60 min, half the time of standard cycling (also works with standard cycling)
  - *For research use only – not for use in diagnostics products*

The above two Master Mixes are preferably recommended for 2019 nCoV assay detection

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Generation 2: TaqMan Array Respiratory Tract Microbiota Card with 2019 nCoV
Use Case: Syndromic Evaluation & Epidemiological Studies
Adenoviruses #1 1
Adenoviruses #2 2
Bocavirus 3
Varicella zoster virus (VZV) 4
Epstein-Barr virus (EBV) 5
Cytomegalovirus 6
Human herpesvirus 6 (HHV-6) 7
Influenza A virus (Pan) 8
Influenza A virus H3 9
Influenza A virus H1-2009 10
IC 18S rRNA 11
Influenza B virus 12
Parainfluenza virus 1 13
Parainfluenza virus 2 14
Parainfluenza virus 3 15
Parainfluenza virus 4 16
Respiratory syncytial virus A 17
Respiratory syncytial virus B 18
Human metapneumovirus 19
Measles 20
Coronavirus 229E 21
Coronavirus HKU1 22
Coronavirus NL63 23
Coronavirus OC43 24
25 Mumps
26 MERS-CoV
27 SARS- CoV
28 Enteroviruses_pan
29 Enteroviruses_D68
30 Rhinoviruses #1
31 Rhinoviruses #2
32 Parechovirus
33 2019-nCoV Assay 1
34 Bordetella (PAN)
35 Bordetella holmesii
36 Bordetella pertussis
37 2019-nCoV Assay 2
38 Chlamydophila pneumoniae
39 Haemophilus Influenzae
40 Klebsiella pneumoniae
41 Legionella pneumophila
42 Moraxella catarrhalis
43 Mycoplasma pneumoniae
44 Staphylococcus aureus
45 Streptococcus pneumoniae
46 Pneumocystis jirovecii
47 2019-nCoV Assay 3
48 IC Rnase P
SKU
Protocol step
SKU name
Unit
Format
4398986 (Generic)
TAC
TaqMan® Array Card, RTM w 2019nCoV
1 Card
Format 48
4441856 (Generic)
PreAmp
TaqMan® PreAmp Pool, RTM w 2019nCoV
1 ml
A15299
PreAmp
TaqPath™ 1-Step RT-qPCR Master Mix, CG
5 x 1 ml
A44359
qPCR
TaqMan® Fast Advanced Master Mix, No UNG
1 ml
A44360
qPCR
TaqMan® Fast Advanced Master Mix, No UNG
5 ml
A30220 (Generic)
Control
Custom Control, RTM w 2019nCoV
250 ul
10^5 cp/ul
The RTM (Respiratory Tract Microbiota) Taqman Array Card, V6 is a customized version of the launched product: Applied Biosystems™ TaqMan® Array Respiratory Tract Microbiota Comprehensive Card (SKU: A41238)
Link: https://www.thermofisher.com/order/catalog/product/A41238
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Generation 1 CoV Detection Product Performance:

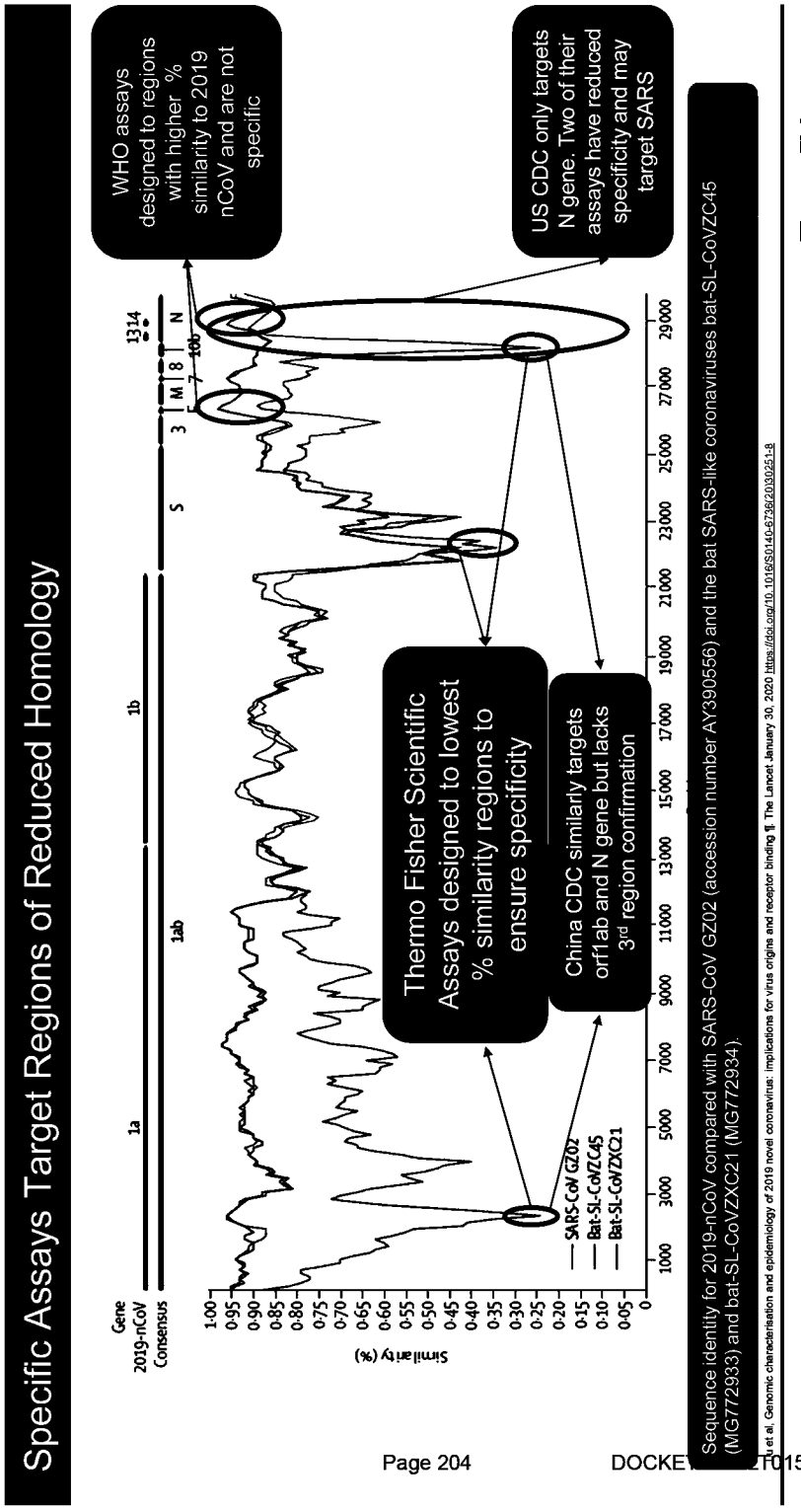
TaqMan 2019nCoV Assay Kit v1
TaqMan 2019nCoV Control Kit v1

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Wuhan Coronavirus nCoV Assay Designs
Assay Offering
Designed assays targeting:
• ORF1ab
• N protein
• S protein (unique offering)
Coverage
Global Initiative on Sharing All Influenza Data (GISAID)
Target 100% of all 52* currently available complete genomes for 2019 nCoV
Specificity
National Center for Biotechnology Information (NCBI)
Specific for 2019 nCoV against:
• 2116 other complete genomes of coronaviruses in NCBI
Highly effective designs for targeting 2019 Novel Coronavirus
*As of January 31, 2020. Genomes are being frequently updated to GISAID because of the extreme interest in this emergence. Percent coverage is being updated by Thermo Fisher Scientific as this information becomes available
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Specific Assays Target Regions of Reduced Homology
A
Strain
Complete genome (%)
Gene region (%)
1ab
1a
1b
S
3
E
M
7
8
10b
N
13
14
Nucleotide sequences
Bat-SL-CoVZC45
87·6
88·9
90·7
86·0
75·2
87·8
98·7
93·4
95·2
88·8
88·5
91·1
89·1
96·7
Bat-SL-CoVZXC21
87·5
88·7
90·3
86·1
74·7
88·9
98·7
93·4
95·2
89·1
88·5
91·2
89·5
96·7
SARS-CoVGZ02
79·0
79·5
75·4
86·3
72·7
75·6
93·5
85·1
74·5
82·1
-
88·1
-
-
Amino acid sequences
Bat-SL-CoVZC45
-
95·6
95·6
95·8
80·2
90·9
100·0
98·6
93·4
87·6
94·2
94·3
73·2
92·9
Bat-SL-CoVZXC21
-
95·2
95·1
95·5
79·6
92·0
100·0
98·6
93·4
88·4
94·2
94·3
73·2
92·9
SARS-CoVGZ02
-
86·2
80·5
95·6
76·2
73·1
94·7
90·1
68·9
85·2
-
90·3
-
-
B
Gene
2019-nCoV Consensus
1a
1ab
1b
S
3
E
M
7
8
10b
N
1314
Orf1ab assay
S gene assay
N gene assay
SARS-CoV GZ02
Bat-SL-CoVZC45
Bat-SL-CoVZXC21
Position
Sequence identity for 2019-nCoV compared with SARS-CoV GZ02 (accession number AY390556) and the bat SARS-like coronaviruses bat-SL-CoVZC45 (MG772933) and bat-SL-CoVZXC21 (MG772934).
* Lu et al. Genomic characterization and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. The Lancet January 30, 2020 https://doi.org/10.1016/S0140-6736(20)30251-8
The three genes targeted in our assays are specifically designed to regions that are strain specific.
• Orf1ab: 1000 – 3000 region
• S gene: 21500 – 23000 region
• N gene: 28000 – 29500 region
When 2019 nCoV is mapped against SARS-CoV, it shows significantly reduced % similarity at three troughs.*
These evident troughs are ideal for identifying 2019 nCoV and are what our assays target.
Competitor RUO assays available in the market also target similarly N and orf1ab, however targeting S gene is unique to Thermo Fisher Scientific and confirmed as an ideal candidate in the Lancet publication by Lu et al.
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Specific Assays Target Regions of Reduced Homology
WHO assays designed to regions with higher % similarity to 2019 nCoV and are not specific
US CDC only targets N gene. Two of their assays have reduced specificity and may target SARS
Thermo Fisher Scientific Assays designed to lowest % similarity regions to ensure specificity
China CDC similarly targets orf1ab and N gene but lacks 3rd region confirmation
Gene
2019-nCoV
Consensus
1a
1b
1ab
S
3
M
8
N
1314
Similarity (%)
SARS-CoV GZ02
Bat-SL-CoVZC45
Bat-SL-CoVZXC21
Sequence identity for 2019-nCoV compared with SARS-CoV GZ02 (accession number AY390556) and the bat SARS-like coronaviruses bat-SL-CoVZC45 (MG772933) and bat-SL-CoVZXC21 (MG772934).
Lu et al. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. The Lancet January 30, 2020
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How our Assays Match Up: In Silico Evaluation

Design Criteria	Thermo Fisher	China CDC	United States CDC	WHO
Target Regions	• Orf1ab* • N Protein* • S Protein*	• Orf1ab (3 assays) • N Protein (Both targets positive for confirmation)	• N Protein (3 assays)	• E Protein (screening) • N Protein (confirmatory) • RdRp-SARS/Wuhan (confirmatory)
Coverage	100% coverage of published genomes	100% coverage of published genomes	100% coverage of published genomes	100% coverage of published genomes
Specificity	All assays are specific	All assays are specific	Two assays (N1 and N3) might generate a signal if SARS or Bat-SARS-CoV are present in higher concentrations.	E and N protein assays map perfectly to hundreds of SARS and other CoV strains. RdRp SARS/Wuhan assay was designed to detect both SARS and 2019nCoV, so it's not specific by design.
Control Assays	RNaseP	No recommendation	RNaseP	No recommendation

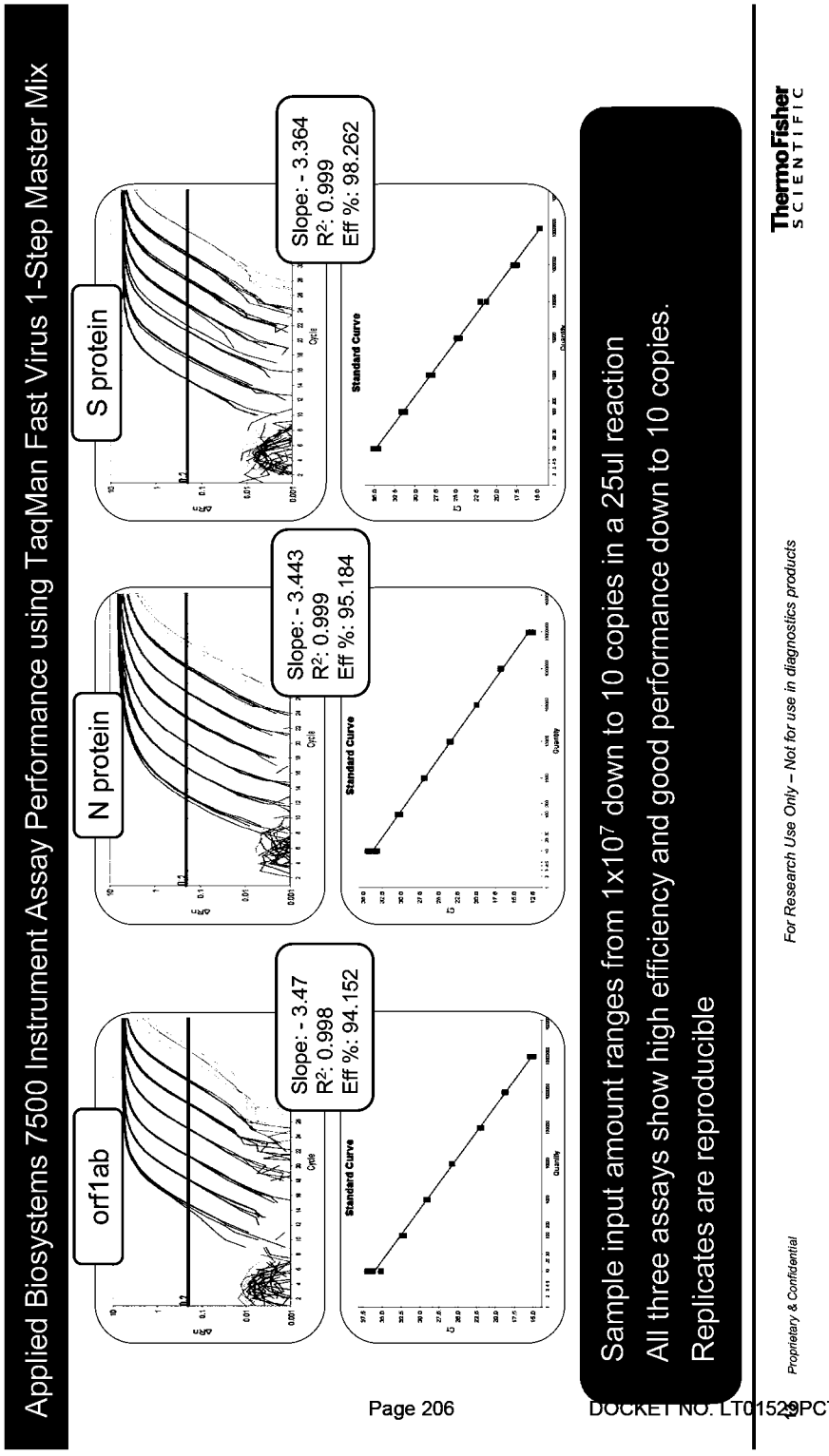
* All of the TaqMan Assays targeting Orf1ab, N Protein and S Protein are unique designs and do not overlap with any designs from US CDC, China CDC or WHO protocols

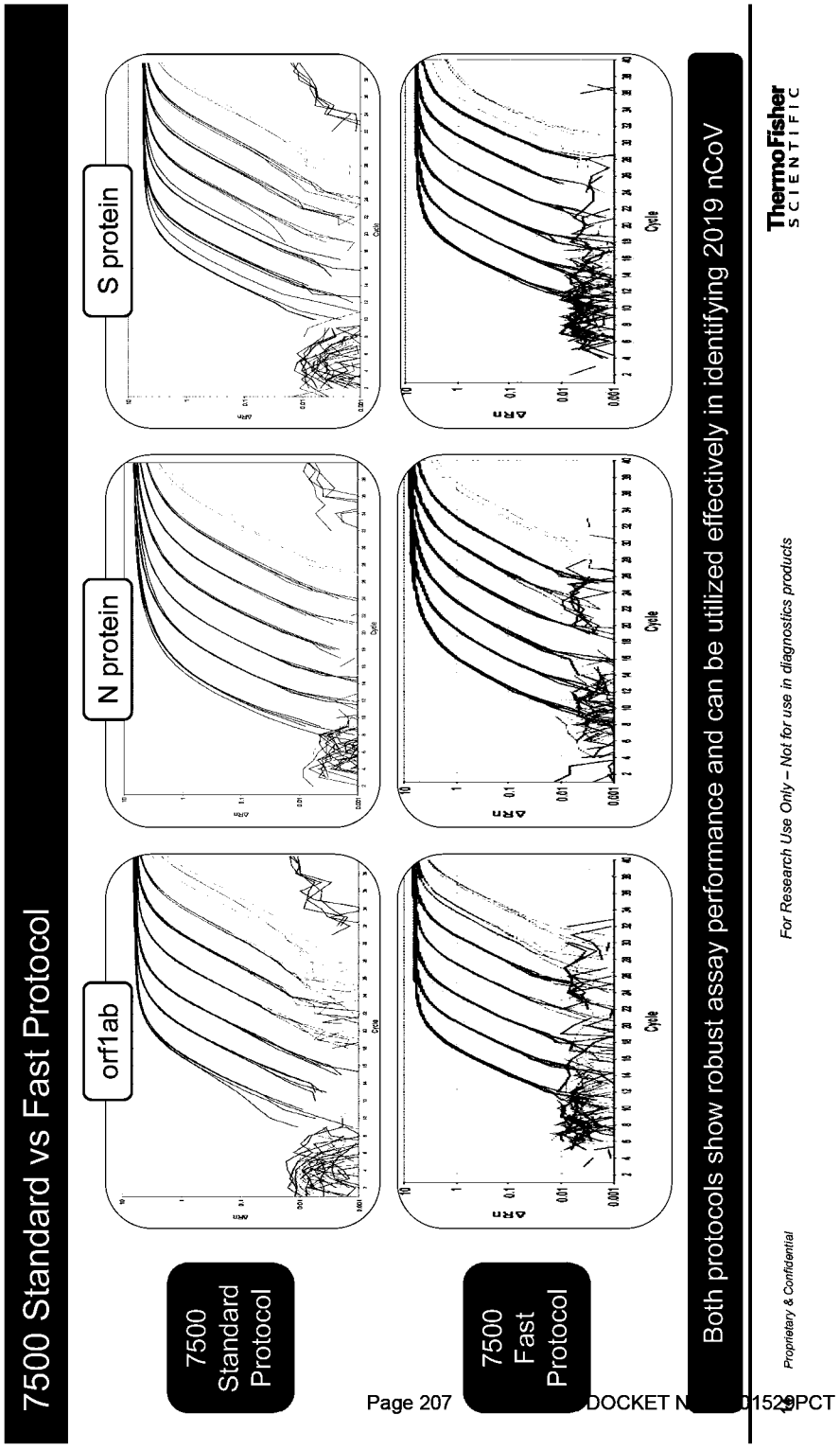
All statements are based on in silico mapping to 52 complete genomes. The mapping was done using our standard mapping tool zpcr3p. All our assays' matches to 52 genomes were perfect – no mismatches.

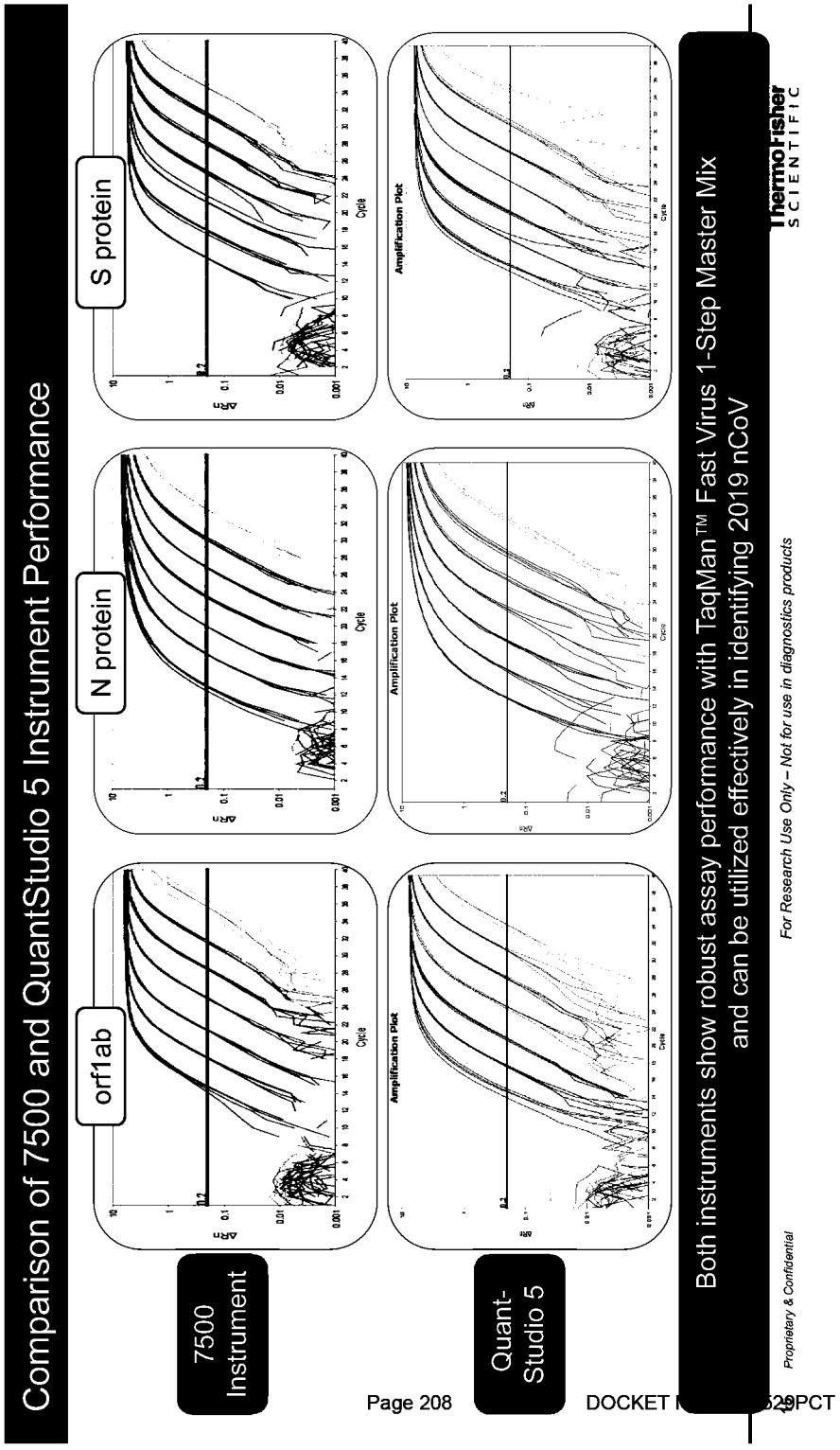
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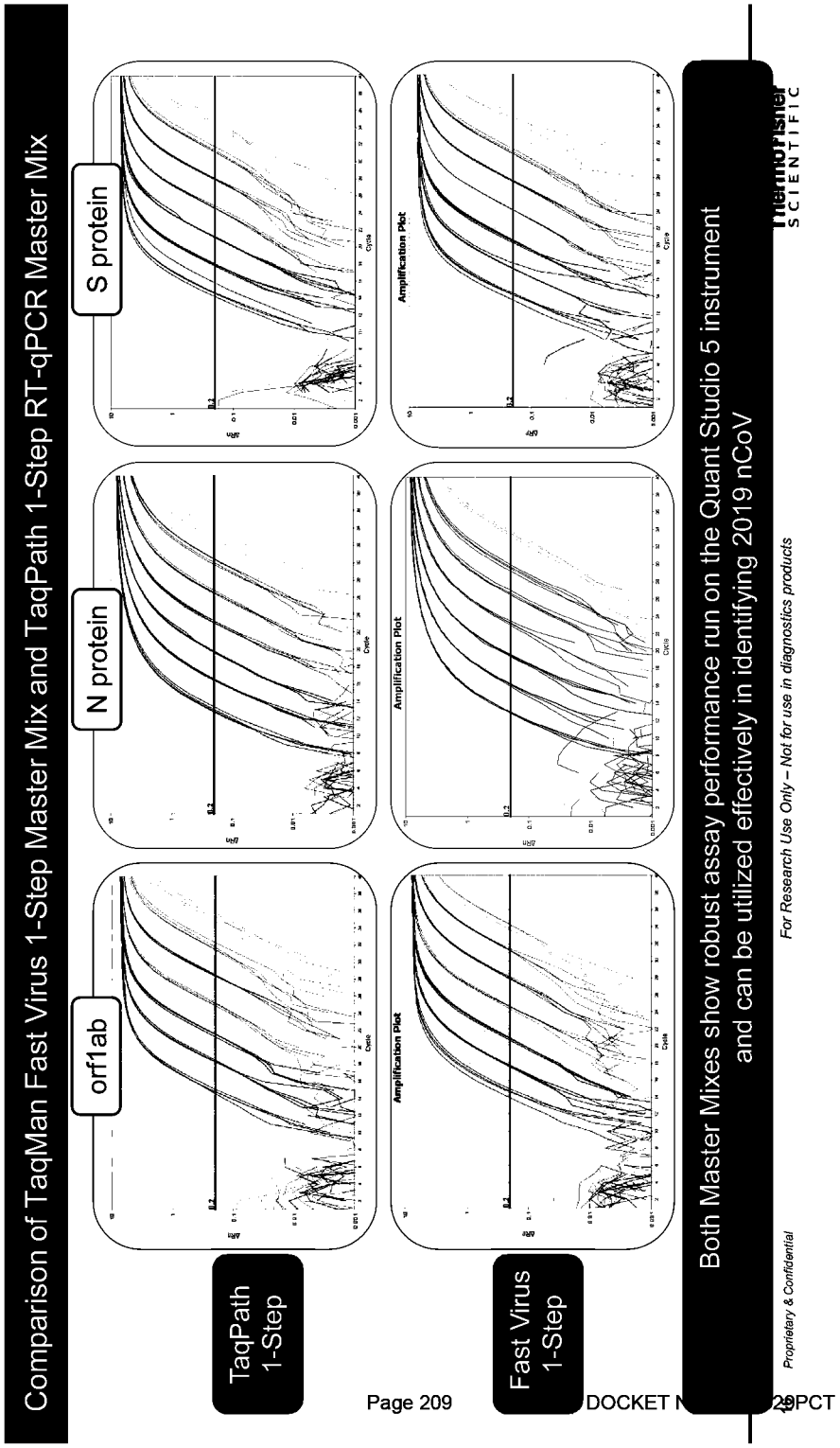
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Applied Biosystems 7500 Instrument Assay Performance using TaqMan Fast Virus 1-Step Master Mix
orf1ab
Slope: - 3.47
R2: 0.998
Eff %: 94.152
N protein
Slope: - 3.443
R2: 0.999
Eff %: 95.184
S protein
Slope: - 3.364
R2: 0.999
Eff %: 98.262
Standard Curve
Sample input amount ranges from 1x10^7 down to 10 copies in a 25ul reaction
All three assays show high efficiency and good performance down to 10 copies.
Replicates are reproducible
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7500 Standard vs Fast Protocol
orf1ab
N protein
S protein
7500 Standard Protocol
7500 Fast Protocol
ΔRn
Cycle
Both protocols show robust assay performance and can be utilized effectively in identifying 2019 nCoV
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Comparison of 7500 and QuantStudio 5 Instrument Performance
orf1ab
N protein
S protein
7500 Instrument
Quant-Studio 5
Amplification Plot
Cycle
Both instruments show robust assay performance with TaqMan™ Fast Virus 1-Step Master Mix and can be utilized effectively in identifying 2019 nCoV
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Comparison of TaqMan Fast Virus 1-Step Master Mix and TaqPath 1-Step RT-qPCR Master Mix
orf1ab
N protein
S protein
TaqPath 1-Step
Fast Virus 1-Step
Amplification Plot
Both Master Mixes show robust assay performance run on the Quant Studio 5 instrument and can be utilized effectively in identifying 2019 nCoV
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Assay Performance Comparison

Target	Applied Biosystems™ Instrument	Applied Biosystems™ MasterMix	Slope	R^2	Efficiency
Orf1ab	7500 Real-Time PCR System	TaqMan® Fast Virus 1-Step Master Mix (Standard)	-3.47	0.998	94.152 %
	QuantStudio™ 5 Real-Time PCR System	TaqMan® Fast Virus 1-Step Master Mix (Standard)	-3.321	0.998	100.022 %
		TaqPath™ 1-Step RT-qPCR Master Mix, CG	-3.385	0.999	97.418 %
N protein	7500 Real-Time PCR System	TaqMan® Fast Virus 1-Step Master Mix (Standard)	-3.443	0.999	95.184 %
	QuantStudio™ 5 Real-Time PCR System	TaqMan® Fast Virus 1-Step Master Mix (Standard)	-3.417	0.999	96.184 %
		TaqPath™ 1-Step RT-qPCR Master Mix, CG	-3.324	0.995	99.909 %
S protein	7500 Real-Time PCR System	TaqMan® Fast Virus 1-Step Master Mix (Standard)	-3.364	0.999	98.262 %
	QuantStudio™ 5 Real-Time PCR System	TaqMan® Fast Virus 1-Step Master Mix (Standard)	-3.408	0.997	96.51 %
		TaqPath™ 1-Step RT-qPCR Master Mix, CG	-3.319	0.998	100.114 %

Assays show good overall performance regardless of instrument and master mix used

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Specificity Data

Sample Tested	Sample Information*	Orf1ab	N Protein	S Protein
Coronavirus OC43	0810024CF	No Amp	No Amp	No Amp
Coronavirus NL63	0810228CF	No Amp	No Amp	No Amp
Coronavirus 229E	0810229CF	No Amp	No Amp	No Amp
Coronavirus HKU1-1	Clinical Isolate	No Amp	No Amp	No Amp
Coronavirus HKU1-2	Clinical Isolate	No Amp	No Amp	No Amp
Flu A H3	0810252CF	No Amp	No Amp	No Amp
Flu A H1N1	08102440F	No Amp	No Amp	No Amp
Flu B	0810255CF	No Amp	No Amp	No Amp
RSV A	0810040ACF	No Amp	No Amp	No Amp
RSV B	0810040CF	No Amp	No Amp	No Amp
Rhinovirus	0810014CF	No Amp	No Amp	No Amp
PIP 1	0810015CF	No Amp	No Amp	No Amp
PIV 2	0810016CF	No Amp	No Amp	No Amp
PIV 3	0810060CF	No Amp	No Amp	No Amp
PIV 4	0810012CFN	No Amp	No Amp	No Amp
Bordetella pertussis	0801460	No Amp	No Amp	No Amp
NTC		No Amp	No Amp	No Amp
2019nCoV Amp control		positive	positive	positive

*Samples with ID's listed were organism controls acquired from ZeptoMetrix Corporation

- The assays were tested with related organisms to showcase specificity of design.
- It was also tested with the amplification control to confirm adequate assay performance.
- No amplification was seen from any near neighbor pathogens, confirming the *in silico* analysis results.
- These assays are specific to 2019-nCoV.

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NTC Performance
Amplification Plot
Amplification Plot
Amplification Plot
Target
Orf1ab
S protein
N protein
CT Undetermined
(12 replicates)
100%
12/12
100%
12/12
100%
12/12
NTC replicates are clean and do not yield any Ct values for the 2019 nCoV assays.
They are all listed as undetermined
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Experiment Setup using TaqPath 1-Step RT-qPCR Master Mix, CG

PCR Reaction Mix Component	Volume per well (25 ul reaction) Standard 96–well 0.2 ml Plates
TaqPath™ 1-Step RT-qPCR Master Mix, CG (4X)	6.25 µL
2019 nCoV TaqMan® Assay (20X) (FAM)	1.25 µL
TaqMan® RNase P Assay, VIC™ dye/QSY™ assay (20X)	1.25 µL
Nuclease-free water	11.25 µL
Purified Sample	5.00 µL
Final Reaction Volume	25.00 µL

Example Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A			ORF1ab RNase P	ORF1ab RNase P			S Protein RNase P	S Protein RNase P			N Protein RNase P	N Protein RNase P
B	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
C	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
D	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
E	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
F	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
G	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
H	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P

NTC Positive Control Test sample

TaqPath™ 1-Step RT-qPCR Master Mix, CG

Catalog Numbers A15299, A15300

Publication Number MAN0007959

Step	Stage	No. of cycles	Temperature	Time
UDG incubation	1	1	25°C	2 minutes
Reverse transcription†	2	1	50°C†	15 minutes
Polymerase activation‡	3	1	95°C‡	2 minutes
Amplification	4	40	95°C	15 seconds
			60°C	60 seconds

† Reverse transcription works best between 48°C and 55°C.

‡ Required for RT inactivation, initial denaturation, and to activate the DNA polymerase.

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Software and Analysis Recommendations

Instrument	Software version	Analysis settings
QuantStudio 5 Real-Time PCR System	QuantStudio Design & Analysis Software v1.5.1	Autobaseline Manual Threshold = 0.2 for all assays
7500 Real-Time PCR System	7500 Software v2.3	Autobaseline Manual Threshold = 0.2 for all assays

Ct Value	Result
Ct < 37 for at least two targets	Positive
37 ≤ Ct < 40 or Undetermined for 2 or more targets	Repeat the test.
Crt = Undetermined for all 3 assays	Negative

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Product Availability & Support

SKU	Product	Availability
A47532	2019nCoV Assay Kit v1 (Combo with three CoV assays & Rnase P control)	Available
A47533	2019nCoV Control Kit v1 (covering three CoV assays)	Available
	Kit Protocol	Feb 20th, 2020
4398986	TaqMan Array Card, RTM v6 w 2019nCoV	Feb 20th, 2020
A30220	RTM Control v2 with 2019nCoV	Feb 20th, 2020
	TAC Protocol (Generic RTM TAC Protocol available)	Feb 28th, 2020

All product and technical inquiries should be directed through current channels

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Experiment Setup using TaqMan Fast Virus 1-Step Master Mix

PCR Reaction Mix Component	Volume per well (25 ul reaction) Standard 96–well 0.2 ml Plates
TaqMan Fast Virus 1-Step RT-qPCR Master Mix, CG (4X)	6.25 µL
2019 nCoV TaqMan® Assay (20X) (FAM)	1.25 µL
TaqMan® RNase P Assay, VIC™ dye/QSY™ assay (20X)	1.25 µL
Nuclease-free water	11.25 µL
Purified Sample	5.00 µL
Final Reaction Volume	25.00 µL

Example Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	[redacted]	[redacted]	ORF1ab RNase P	ORF1ab RNase P	[redacted]	[redacted]	S Protein RNase P	S Protein RNase P	[redacted]	[redacted]	N Protein RNase P	N Protein RNase P
B	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
C	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
D	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
E	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
F	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
G	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
H	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P

NTC — Positive Control — Test sample

TaqMan® Fast Virus 1-Step Master Mix

Catalog Numbers 4444432, 4444434, 4444436

Publication Part Number 4453800

Step	Stage	No. of cycles	Temperature	Time
Reverse transcription	1	1	50°C†	5 minutes
RT inactivation/initial denaturation	2	1	95°C	20 seconds
Amplification	3	40	95°C	15 seconds
			60°C	60 seconds

† Reverse transcription works best between 48°C and 55°C.

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APPENDIX 6

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Genetic Analysis Solutions for 2019-nCoV (novel Coronavirus)
DOCKET NO. LTC
February 18th, 2020
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Coronavirus Family – Global Impact in Humans

- Coronaviruses are named for the crown-like spikes on their surface. There are four main sub-groupings of coronaviruses, known as alpha, beta, gamma, and delta. Human coronaviruses were first identified in the mid-1960s. The seven coronaviruses that can infect humans are:
- Common human coronaviruses
  - 229E (alpha coronavirus)
  - NL63 (alpha coronavirus)
  - OC43 (beta coronavirus)
  - HKU1 (beta coronavirus)

People around the world commonly get infected with human coronaviruses 229E, NL63, OC43, and HKU1

- Coronaviruses can infect animals and evolve and undergo zoonosis to new human coronavirus. Coronaviruses that have crossed over to humans are:
  - MERS-CoV (the beta coronavirus that causes Middle East Respiratory Syndrome, or MERS)
  - SARS-CoV (the beta coronavirus that causes severe acute respiratory syndrome, or SARS)
  - 2019 Novel Coronavirus (2019-nCoV)

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Applied Biosystems TaqMan qPCR Assays for Coronaviruses Family

Product type	SKU	Assay ID	Assay name
TaqMan Gene Expression Assay	4331182	Vi06439671_s1	Human Coronavirus 229E
TaqMan Gene Expression Assay	4331182	Vi06439674_s1	Human Coronavirus HKU1
TaqMan Gene Expression Assay	4331182	Vi06439673_s1	Human Coronavirus NL63
TaqMan Gene Expression Assay	4331182	Vi06439646_s1	Human Coronavirus OC43
TaqMan Gene Expression Assay	4331182	Vi06439644_s1	Middle East Respiratory Syndrome coronavirus (MERS)
TaqMan Gene Expression Assay	4331182	Vi06439634_s1	Severe Acute Respiratory Syndrome coronavirus (SARS)

Above assays are orderable on the web for customers
Primary use case for a customer is to differentiate samples with known coronaviruses
Available in small, medium and large sizes (similar to other inventoried
Applied Biosystems™ TaqMan® Gene Expression Assays)

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Generation 1: 2019-nCoV Assay Kit and Control

SKU	SKU name	Unit	Product description	Unit size	Status
A47532	TaqMan 2019nCoV Assay Kit v1	1 wet tube	2019nCoV Assay (Gene Orf-1ab) – FAM, 20X	50 reactions (75 ul)	Available
		1 wet tube	2019nCoV Assay (Gene S protein) – FAM, 20X	50 reactions (75 ul)	
		1 wet tube	2019nCoV Assay (Gene N protein) – FAM, 20X	50 reactions (75 ul)	
		1 wet tube	RNase P – VIC, 20X	150 reactions (225 ul)	
A47533	TaqMan 2019nCoV Control Kit v1	1 wet tube	2019nCoV DNA Control (covering Gene Orf-1ab, Gene S protein and Gene N protein, RNase P)	50 ul (Conc 1X10^4 copies/uL)	Available

Use Case: Evaluation of respiratory samples in laboratory settings

- Kit assays target all 71 currently available complete genomes at GISAID and do not target any of the 2116 complete genomes of other coronaviruses currently available at NCBI

Larger units of assays and controls available on special order

Applied Biosystems™ TaqMan® 2019nCoV Assay Kit v1 & TaqMan® 2019nCoV Control Kit v1 are manufactured at different physical locations

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2019 nCoV Synthetic Control Information
orf1ab
N protein
S protein
RNase P
Xeno
To test performance of assays targeting 2019 nCoV, we provide a synthetic plasmid control.
The control includes the assay target insert for all three targets:
• Gene orf1ab
• Gene S protein
• Gene N protein
It also incorporates control target:
• RNaseP
• Xeno (included, if needed as a contamination marker)
The control is provided at 1x10⁴ copies/ul (Digitally quantified)
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Master Mixes Recommended for 2019 nCoV Assay Detection

- **Applied Biosystems™ TaqPath™ 1-Step RT-qPCR Master Mix, CG (PN - A15299)**
  - All the advantages of Applied Biosystems™ TaqMan® Fast Virus 1-step Master Mix
  - Includes UNG and dUTP for carryover contamination control
  - Enhanced quality control measures for even better lot to lot consistency
  - For use primarily in the US, as written into key protocols by the US CDC
  - *General purpose reagent*
- **Applied Biosystems™ TaqMan® Fast Virus 1-step Master Mix (PN - 4444434, 4444432, 4444436)**
  - Highly concentrated (4X) master mix – ability to add more sample for improved sensitivity
  - Exhibits inhibitor tolerance, especially those found in difficult sample types, provides greater accuracy
  - One-tube master mix for improved efficiency and ease of use
  - Optimized for multiplexing (up to 3 targets) reactions and with exogenous/endogenous internal controls
  - Fast cycling – provides results in ~60 min, half the time of standard cycling (also works with standard cycling)
  - *For research use only – not for use in diagnostics products*

The above two Master Mixes are preferably recommended for 2019 nCoV assay detection

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Generation 2: TaqMan Array Respiratory Tract Microbiota Card with 2019 nCoV

Use Case: Syndromic Evaluation & Epidemiological Studies

Adenoviruses #1	1	25	Mumps	SKU	Protocol step	SKU name	Unit	Format
Adenoviruses #2	2	26	MERS-CoV	4398986 (Generic)	TAC	TaqMan® Array Card, RTM w 2019nCoV	1 Card	Format 48
Bocavirus	3	27	SARS- CoV					
Varicella zoster virus (VZV)	4	28	Enteroviruses_pan	4441856 (Generic)	PreAmp	TaqMan® PreAmp Pool, RTM w 2019nCoV	1 ml	
Epstein-Barr virus (EBV)	5	29	Enteroviruses_D68	A15299	PreAmp	TaqPath™ 1-Step RT-qPCR Master Mix, CG	5 x 1 ml	
Cytomegalovirus	6	30	Rhinoviruses #1	A44359	qPCR	TaqMan® Fast Advanced Master Mix, No UNG	1 ml	
Human herpesvirus 6 (HHV-6)	7	31	Rhinoviruses #2	A44360	qPCR	TaqMan® Fast Advanced Master Mix, No UNG	5 ml	
Influenza A virus (Pan)	8	32	Parechovirus	A30220 (Generic)	Control	Custom Control, RTM w 2019nCoV	250 ul	10^5 cp/ul
Influenza A virus H3	9	33	**2019-nCoV Assay 1**					
Influenza A virus H1-2009	10	34	Bordetella (PAN)					
IC 18S rRNA	11	35	Bordetella holmesii					
Influenza B virus	12	36	Bordetella pertussis					
Parainfluenza virus 1	13	37	**2019-nCoV Assay 2**					
Parainfluenza virus 2	14	38	Chlamydophila pneumoniae					
Parainfluenza virus 3	15	39	Haemophilus Influenzae					
Parainfluenza virus 4	16	40	Klebsiella pneumoniae					
Respiratory syncytial virus A	17	41	Legionella pneumophila					
Respiratory syncytial virus B	18	42	Moraxella catarrhalis					
Human metapneumovirus	19	43	Mycoplasma pneumoniae					
Measles	20	44	Staphylococcus aureus					
Coronavirus 229E	21	45	Streptococcus pneumoniae					
Coronavirus HKU1	22	46	Pneumocystis jirovecii					
Coronavirus NL63	23	47	**2019-nCoV Assay 3**					
Coronavirus OC43	24	48	**IC Rnase P**					

The **RTM** (Respiratory Tract Microbiota) Taqman Array Card, V6 is a customized version of the launched product: Applied Biosystems™ TaqMan® Array Respiratory Tract Microbiota Comprehensive Card (SKU: **A41238**)
Link: https://www.thermofisher.com/order/catalog/product/A41238

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Generation 1 CoV Detection Product Performance:

TaqMan 2019nCoV Assay Kit v1
TaqMan 2019nCoV Control Kit v1

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Wuhan Coronavirus nCoV Assay Designs
Assay Offering
Designed assays targeting:
• ORF1ab
• N protein
• S protein (unique offering)
Coverage
Global Initiative on Sharing All Influenza Data (GISAID)
Target 100% of all 52* currently available complete genomes for 2019 nCoV
Specificity
National Center for Biotechnology Information (NCBI)
Specific for 2019 nCoV against:
• 2116 other complete genomes of coronaviruses in NCBI
Highly effective designs for targeting 2019 Novel Coronavirus
*As of January 31, 2020. Genomes are being frequently updated to GISAID because of the extreme interest in this emergence. Percent coverage is being updated by Thermo Fisher Scientific as this information becomes available
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Specific Assays Target Regions of Reduced Homology

The three genes targeted in our assays are specifically designed to regions that are strain specific.

- Orf1ab: 1000 – 3000 region
- S gene: 21500 – 23000 region
- N gene: 28000 – 29500 region

When 2019 nCoV is mapped against SARS-CoV, it shows significantly reduced % similarity at three troughs.*

These evident troughs are ideal for identifying 2019 nCoV and are what our assays target.

Competitor RUO assays available in the market also target similarly N and orf1ab, however targeting S gene is unique to Thermo Fisher Scientific and confirmed as an ideal candidate in the Lancet publication by Lu et al.

A

	Strain	Complete genome (%)	Gene region (%)												
			1ab	1a	1b	S	3	E	M	7	8	10b	N	13	14
Nucleotide sequences	Bat-SL-CoVZC45	87·6	88·9	90·7	86·0	75·2	87·8	98·7	93·4	95·2	88·8	88·5	91·1	89·1	96·7
	Bat-SL-CoVZXC21	87·5	88·7	90·3	86·1	74·7	88·9	98·7	93·4	95·2	89·1	88·5	91·2	89·5	96·7
	SARS-CoVGZ02	79·0	79·5	75·4	86·3	72·7	75·6	93·5	85·1	74·5	82·1	..	88·1	..	..
Amino acid sequences	Bat-SL-CoVZC45	..	95·6	95·6	95·8	80·2	90·9	100·0	98·6	93·4	87·6	94·2	94·3	73·2	92·9
	Bat-SL-CoVZXC21	..	95·2	95·1	95·5	79·6	92·0	100·0	98·6	93·4	88·4	94·2	94·3	73·2	92·9
	SARS-CoVGZ02	..	86·2	80·5	95·6	76·2	73·1	94·7	90·1	68·9	85·2	..	90·3	..	..

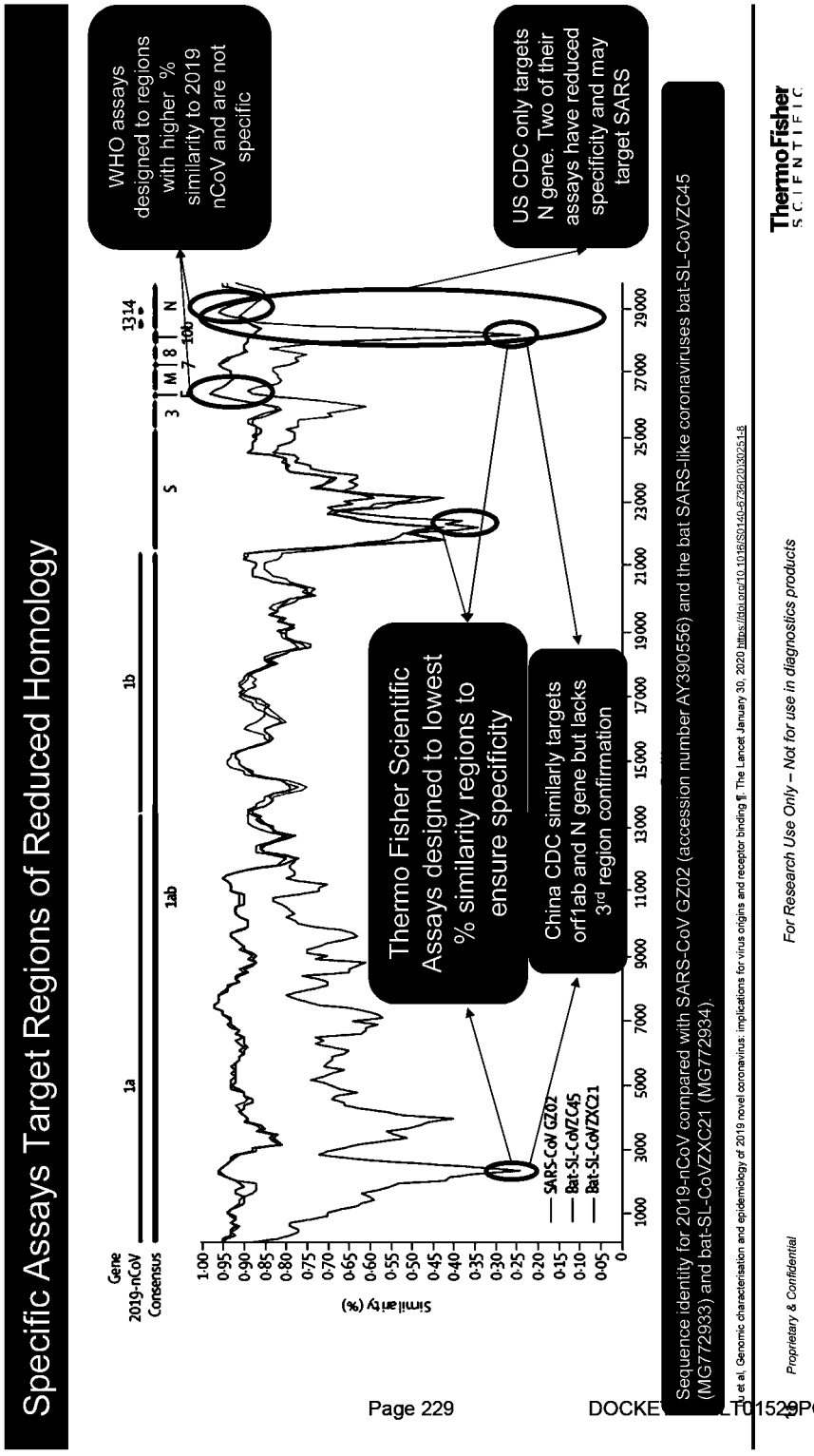
B

Sequence identity for 2019-nCoV compared with SARS-CoV GZ02 (accession number AY390556) and the bat SARS-like coronaviruses bat-SL-CoVZC45 (MG772933) and bat-SL-CoVZXC21 (MG772934).

* Lu et al. Genomic characterization and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. The Lancet January 30, 2020 https://doi.org/10.1016/S0140-6736(20)30251-8

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Specific Assays Target Regions of Reduced Homology
WHO assays designed to regions with higher % similarity to 2019 nCoV and are not specific
US CDC only targets N gene. Two of their assays have reduced specificity and may target SARS
Thermo Fisher Scientific Assays designed to lowest % similarity regions to ensure specificity
China CDC similarly targets orf1ab and N gene but lacks 3rd region confirmation
Gene
2019-nCoV
Consensus
1a
1b
1ab
S
3
M
8
N
1314
SARS-CoV GZ02
Bat-SL-CoVZC45
Bat-SL-CoVZXC21
Similarity (%)
Sequence identity for 2019-nCoV compared with SARS-CoV GZ02 (accession number AY390556) and the bat SARS-like coronaviruses bat-SL-CoVZC45 (MG772933) and bat-SL-CoVZXC21 (MG772934).
Wu et al. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding ¶. The Lancet January 30, 2020 https://doi.org/10.1016/S0140-6736(20)30251-8
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How our Assays Match Up: In Silico Evaluation

Design Criteria	Thermo Fisher	China CDC	United States CDC	WHO
Target Regions	• Orf1ab* • N Protein* • S Protein*	• Orf1ab (3 assays) • N Protein (Both targets positive for confirmation)	• N Protein (3 assays)	• E Protein (screening) • N Protein (confirmatory) • RdRp-SARS/Wuhan (confirmatory)
Coverage	100% coverage of published genomes	100% coverage of published genomes	100% coverage of published genomes	100% coverage of published genomes
Specificity	All assays are specific	All assays are specific	Two assays (N1 and N3) might generate a signal if SARS or Bat-SARS-CoV are present in higher concentrations.	E and N protein assays map perfectly to hundreds of SARS and other CoV strains. RdRp SARS/Wuhan assay was designed to detect both SARS and 2019nCoV, so it's not specific by design.
Control Assays	RNaseP	No recommendation	RNaseP	No recommendation

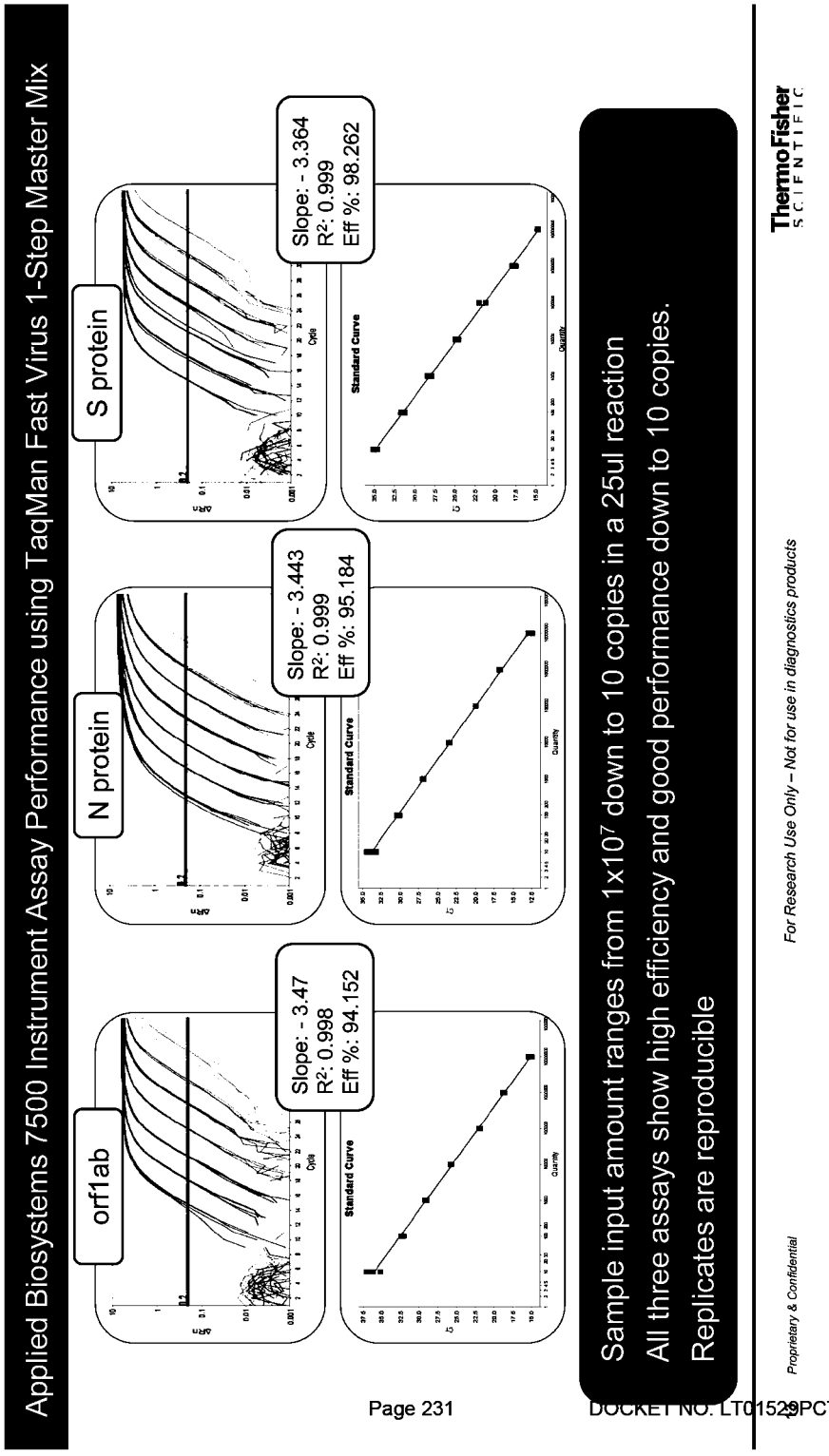
* All of the TaqMan Assays targeting Orf1ab, N Protein and S Protein are unique designs and do not overlap with any designs from US CDC, China CDC or WHO protocols

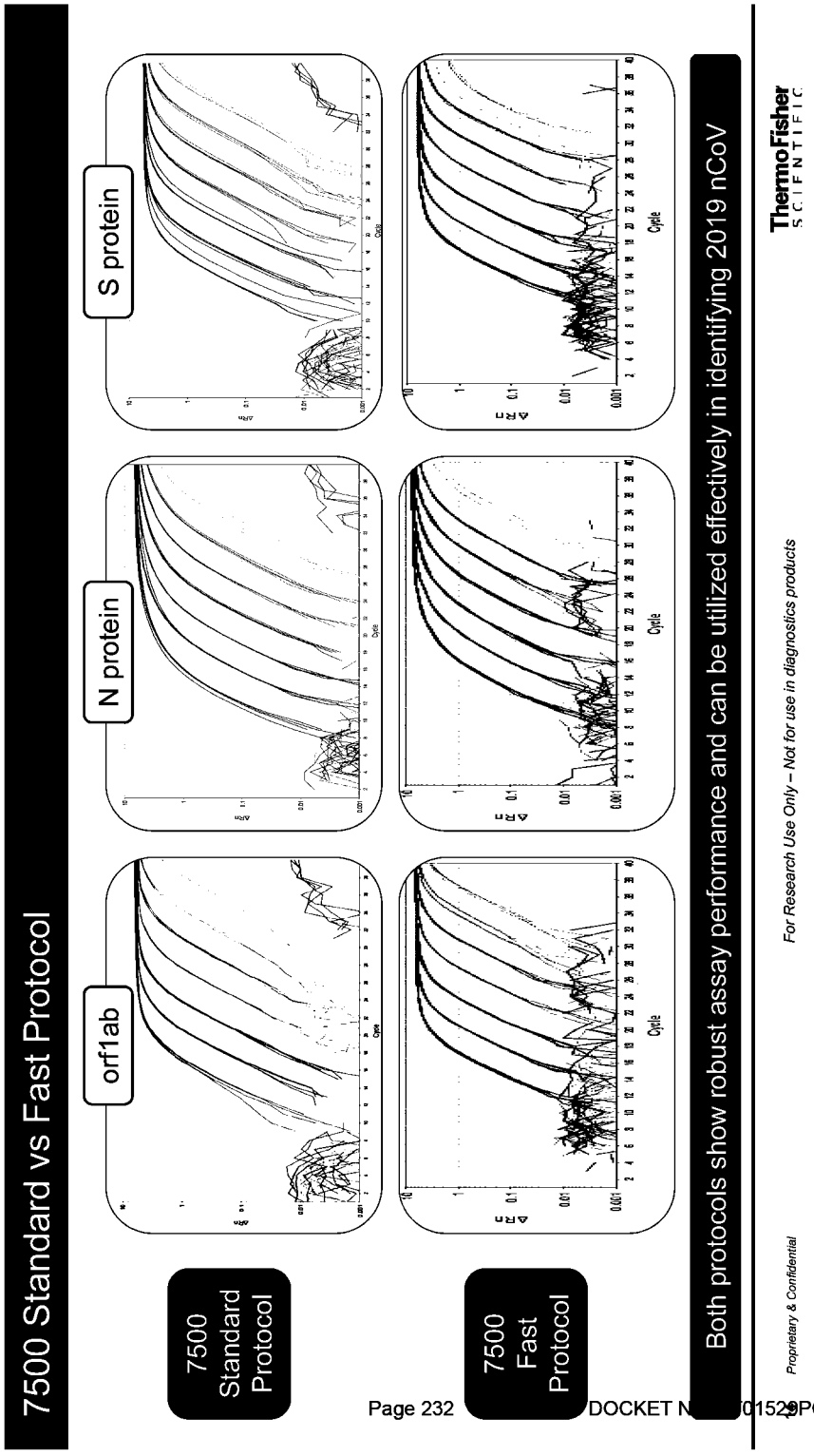
All statements are based on in silico mapping to 52 complete genomes. The mapping was done using our standard mapping tool zpcr3p. All our assays' matches to 52 genomes were perfect – no mismatches.

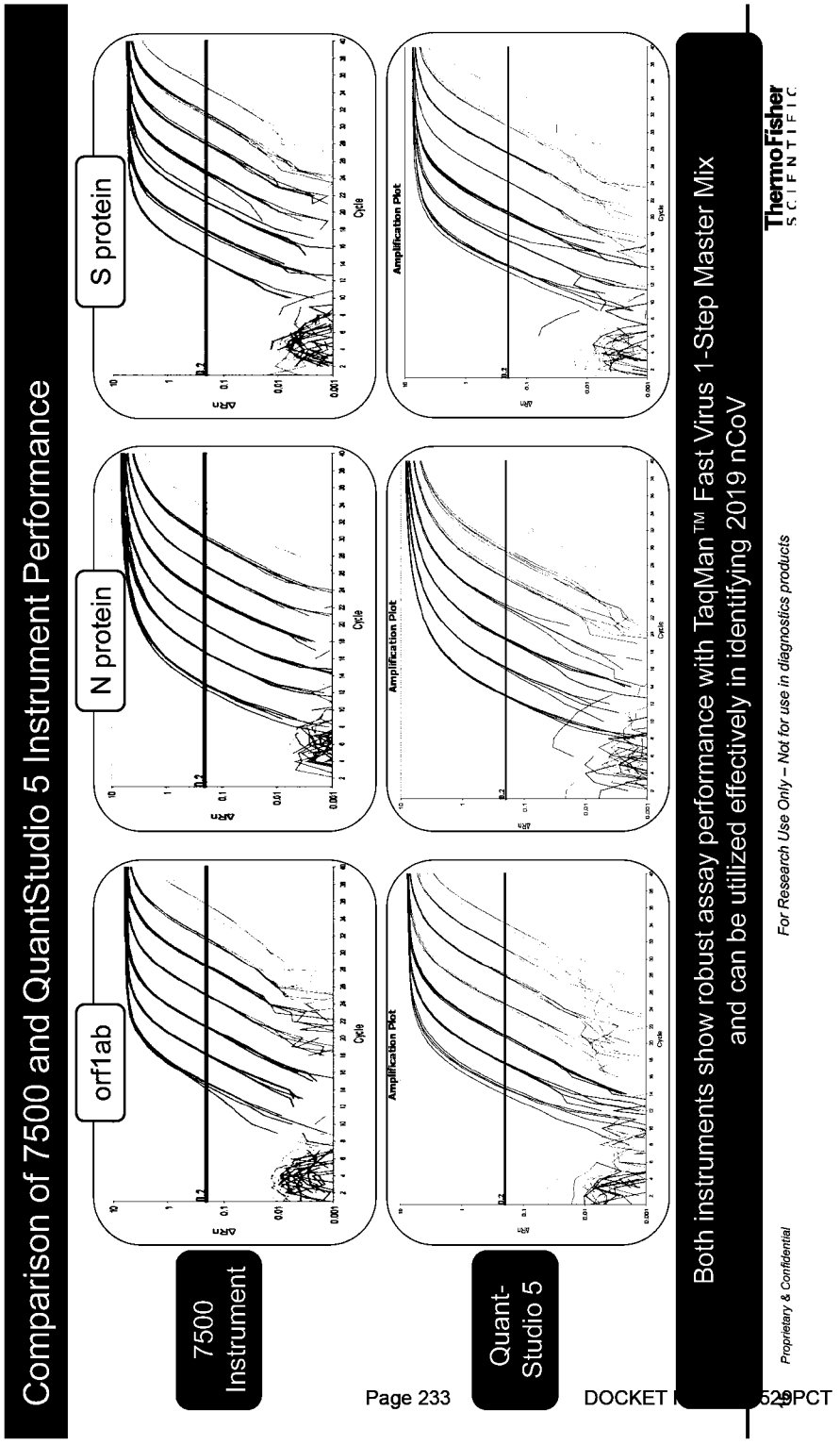
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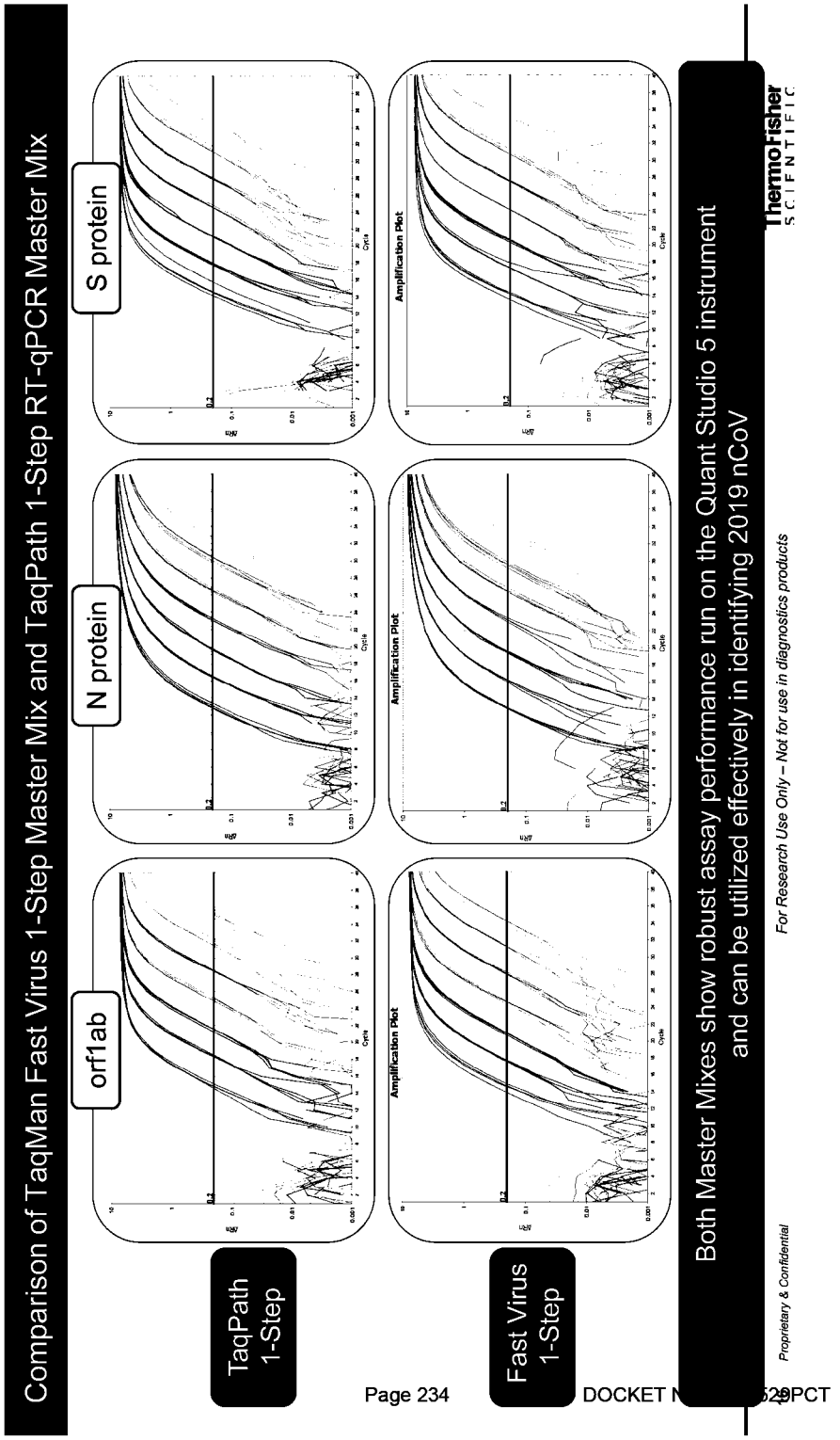
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Applied Biosystems 7500 Instrument Assay Performance using TaqMan Fast Virus 1-Step Master Mix
orf1ab
Slope: - 3.47
R2: 0.998
Eff %: 94.152
N protein
Slope: - 3.443
R2: 0.999
Eff %: 95.184
S protein
Slope: - 3.364
R2: 0.999
Eff %: 98.262
Standard Curve
Sample input amount ranges from 1x10^7 down to 10 copies in a 25ul reaction
All three assays show high efficiency and good performance down to 10 copies.
Replicates are reproducible
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7500 Standard vs Fast Protocol
orf1ab
N protein
S protein
7500 Standard Protocol
7500 Fast Protocol
Cycle
Both protocols show robust assay performance and can be utilized effectively in identifying 2019 nCoV
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Comparison of 7500 and QuantStudio 5 Instrument Performance
orf1ab
N protein
S protein
7500 Instrument
Quant-Studio 5
Amplification Plot
Cycle
Both instruments show robust assay performance with TaqMan™ Fast Virus 1-Step Master Mix and can be utilized effectively in identifying 2019 nCoV
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Comparison of TaqMan Fast Virus 1-Step Master Mix and TaqPath 1-Step RT-qPCR Master Mix
orf1ab
N protein
S protein
TaqPath 1-Step
Fast Virus 1-Step
Amplification Plot
Both Master Mixes show robust assay performance run on the Quant Studio 5 instrument and can be utilized effectively in identifying 2019 nCoV
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Assay Performance Comparison

Target	Applied Biosystems™ Instrument	Applied Biosystems™ MasterMix	Slope	R^2	Efficiency
Orf1ab	7500 Real-Time PCR System	TaqMan® Fast Virus 1-Step Master Mix (Standard)	-3.47	0.998	94.152 %
	QuantStudio™ 5 Real-Time PCR System	TaqMan® Fast Virus 1-Step Master Mix (Standard)	-3.321	0.998	100.022 %
		TaqPath™ 1-Step RT-qPCR Master Mix, CG	-3.385	0.999	97.418 %
N protein	7500 Real-Time PCR System	TaqMan® Fast Virus 1-Step Master Mix (Standard)	-3.443	0.999	95.184 %
	QuantStudio™ 5 Real-Time PCR System	TaqMan® Fast Virus 1-Step Master Mix (Standard)	-3.417	0.999	96.184 %
		TaqPath™ 1-Step RT-qPCR Master Mix, CG	-3.324	0.995	99.909 %
S protein	7500 Real-Time PCR System	TaqMan® Fast Virus 1-Step Master Mix (Standard)	-3.364	0.999	98.262 %
	QuantStudio™ 5 Real-Time PCR System	TaqMan® Fast Virus 1-Step Master Mix (Standard)	-3.408	0.997	96.51 %
		TaqPath™ 1-Step RT-qPCR Master Mix, CG	-3.319	0.998	100.114 %

Assays show good overall performance regardless of instrument and master mix used

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Specificity Data

Sample Tested	Sample Information*	Orf1ab	N Protein	S Protein
Coronavirus OC43	0810024CF	No Amp	No Amp	No Amp
Coronavirus NL63	0810228CF	No Amp	No Amp	No Amp
Coronavirus 229E	0810229CF	No Amp	No Amp	No Amp
Coronavirus HKU1-1	Clinical Isolate	No Amp	No Amp	No Amp
Coronavirus HKU1-2	Clinical Isolate	No Amp	No Amp	No Amp
Flu A H3	0810252CF	No Amp	No Amp	No Amp
Flu A H1N1	08102444CF	No Amp	No Amp	No Amp
Flu B	0810255CF	No Amp	No Amp	No Amp
RSV A	0810040ACF	No Amp	No Amp	No Amp
RSV B	0810040CF	No Amp	No Amp	No Amp
Rhinovirus	0810014CF	No Amp	No Amp	No Amp
PIP 1	0810015CF	No Amp	No Amp	No Amp
PIV 2	0810016CF	No Amp	No Amp	No Amp
PIV 3	0810060CF	No Amp	No Amp	No Amp
PIV 4	0810012CFN	No Amp	No Amp	No Amp
Bordetella pertussis	0801460	No Amp	No Amp	No Amp
NTC		No Amp	No Amp	No Amp
2019nCoV Amp control		positive	positive	positive

*Samples with ID's listed were organism controls acquired from ZeptoMetrix Corporation

- The assays were tested with related organisms to showcase specificity of design.
- It was also tested with the amplification control to confirm adequate assay performance.
- No amplification was seen from any near neighbor pathogens, confirming the *in silico* analysis results.
- These assays are specific to 2019-nCoV.

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NTC Performance
Amplification Plot
Amplification Plot
Amplification Plot
Target
Orf1ab
S protein
N protein
CT Undetermined
(12 replicates)
100%
12/12
100%
12/12
100%
12/12
NTC replicates are clean and do not yield any Ct values for the 2019 nCoV assays.
They are all listed as undetermined
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Experiment Setup using TaqPath 1-Step RT-qPCR Master Mix, CG

PCR Reaction Mix Component	Volume per well (25 ul reaction) Standard 96–well 0.2 ml Plates
TaqPath™ 1-Step RT-qPCR Master Mix, CG (4X)	6.25 µL
2019 nCoV TaqMan® Assay (20X) (FAM)	1.25 µL
TaqMan® RNase P Assay, VIC™ dye/QSY™ assay (20X)	1.25 µL
Nuclease-free water	11.25 µL
Purified Sample	5.00 µL
Final Reaction Volume	25.00 µL

TaqPath™ 1-Step RT-qPCR Master Mix, CG

Catalog Numbers A15299, A15300

Publication Number MAN0007959

Step	Stage	No. of cycles	Temperature	Time
UNG incubation	1	1	25°C	2 minutes
Reverse transcription†	2	1	50°C†	15 minutes
Polymerase activation‡	3	1	95°C‡	2 minutes
Amplification	4	40	95°C	15 seconds
			60°C	60 seconds

† Reverse transcription works best between 48°C and 55°C.

‡ Required for RT inactivation, initial denaturation, and to activate the DNA polymerase.

Example Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	[redacted]	[redacted]	ORF1ab	ORF1ab	[redacted]	[redacted]	S Protein	S Protein	[redacted]	[redacted]	N Protein	N Protein
	[redacted]	[redacted]	RNase P	RNase P	[redacted]	[redacted]	RNase P	RNase P	[redacted]	[redacted]	RNase P	RNase P
B	ORF1ab	ORF1ab	ORF1ab	ORF1ab	S Protein	S Protein	S Protein	S Protein	N Protein	N Protein	N Protein	N Protein
	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P
C	ORF1ab	ORF1ab	ORF1ab	ORF1ab	S Protein	S Protein	S Protein	S Protein	N Protein	N Protein	N Protein	N Protein
	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P
D	ORF1ab	ORF1ab	ORF1ab	ORF1ab	S Protein	S Protein	S Protein	S Protein	N Protein	N Protein	N Protein	N Protein
	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P
E	ORF1ab	ORF1ab	ORF1ab	ORF1ab	S Protein	S Protein	S Protein	S Protein	N Protein	N Protein	N Protein	N Protein
	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P
F	ORF1ab	ORF1ab	ORF1ab	ORF1ab	S Protein	S Protein	S Protein	S Protein	N Protein	N Protein	N Protein	N Protein
	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P
G	ORF1ab	ORF1ab	ORF1ab	ORF1ab	S Protein	S Protein	S Protein	S Protein	N Protein	N Protein	N Protein	N Protein
	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P
H	ORF1ab	ORF1ab	ORF1ab	ORF1ab	S Protein	S Protein	S Protein	S Protein	N Protein	N Protein	N Protein	N Protein
	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P	RNase P

NTC — Positive Control — Test sample

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Software and Analysis Recommendations

Instrument	Software version	Analysis settings
QuantStudio 5 Real-Time PCR System	QuantStudio Design & Analysis Software v1.5.1	Autobaseline Manual Threshold = 0.2 for all assays
7500 Real-Time PCR System	7500 Software v2.3	Autobaseline Manual Threshold = 0.2 for all assays

Ct Value	Result
Ct < 37 for at least two targets	Positive
37 ≤ Ct < 40 or Undetermined for 2 or more targets	Repeat the test.
Crt. = Undetermined for all 3 assays	Negative

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Product Availability & Support

SKU	Product	Availability
A47532	2019nCoV Assay Kit v1 (Combo with three CoV assays & Rnase P control)	Available
A47533	2019nCoV Control Kit v1 (covering three CoV assays)	Available
	Kit Protocol	Feb 20th, 2020
4398986	TaqMan Array Card, RTM v6 w 2019nCoV	Feb 20th, 2020
A30220	RTM Control v2 with 2019nCoV	Feb 20th, 2020
	TAC Protocol (Generic RTM TAC Protocol available)	Feb 28th, 2020

All product and technical inquiries should be directed through current channels

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Appendix

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Experiment Setup using TaqMan Fast Virus 1-Step Master Mix

PCR Reaction Mix Component	Volume per well (25 ul reaction) Standard 96–well 0.2 ml Plates
TaqMan Fast Virus 1-Step RT-qPCR Master Mix, CG (4X)	6.25 µL
2019 nCoV TaqMan® Assay (20X) (FAM)	1.25 µL
TaqMan® RNase P Assay, VIC™ dye/QSY™ assay (20X)	1.25 µL
Nuclease-free water	11.25 µL
Purified Sample	5.00 µL
Final Reaction Volume	25.00 µL

TaqMan® Fast Virus 1-Step Master Mix

Catalog Numbers 4444432, 4444434, 4444436

Publication Part Number 4453800

Step	Stage	No. of cycles	Temperature	Time
Reverse transcription	1	1	50°C†	5 minutes
RT inactivation/initial denaturation	2	1	95°C	20 seconds
Amplification	3	40	95°C	15 seconds
			60°C	60 seconds

† Reverse transcription works best between 48°C and 55°C.

Example Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	[redacted]	[redacted]	ORF1ab RNase P	ORF1ab RNase P	[redacted]	[redacted]	S Protein RNase P	S Protein RNase P	[redacted]	[redacted]	N Protein RNase P	N Protein RNase P
B	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
C	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
D	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
E	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
F	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
G	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P
H	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	ORF1ab RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	S Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P	N Protein RNase P

NTC | Positive Control | Test sample

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APPENDIX 7

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TaqMan™ 2019-nCoV Assay Kit v1
Instrument-specific data:
QuantStudio 5 Dx Real-Time PCR System (QS5 Dx)
QuantStudio Dx Real-Time Instrument (QS Dx)
QuantStudio 7 Pro Real-Time PCR System (QS7 Pro)
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Instrument Configurations

Instrument	Instrument Software	Data Analysis Software	Block	Ramp	Master Mix	Volume (ul)
QS Dx	QuantStudio Dx IVD Software v1	QuantStudio DX Software v1	96 well 0.1 ml	Standard	Fast Virus 1-Step	25
QS5 Dx	QuantStudio 5 Dx Software	QuantStudio 5 Dx Software	96 well 0.2 ml	Standard	Fast Virus 1-Step	25
QS7 Pro	QuantStudio Design & Analysis Software v2.3	QuantStudio Design & Analysis Software v2.3	96 well 0.2 ml	Standard	Fast Virus 1-Step	25

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Contents and Storage

Contents	Dye	Amount[1]	Concentration	Storage
2019-nCoV (Orf-1ab) (Tube 1)	FAM™ dye	75 µL	20X	-30°C to -10°C
2019-nCoV (S Protein) (Tube 2)	FAM™ dye	75 µL	20X	
2019-nCoV (N Protein) (Tube 3)	FAM™ dye	75 µL	20X	
RNase P Assay (Tube 4)	VIC™ dye	250 µL	20X	

[1] Sufficient for 50 x 25-µL reactions.

Reaction Setup

3 reactions per sample (with added RNase P in duplex)
1 reaction per nCoV assay (ORF1ab, N Protein, S Protein)

1. For each COVID-19 virus Assay, combine the following components for the number of reactions, plus 10% overage:

Component	Volume per reaction
Master Mix (4X)	6.25 µL
COVID-19 virus Assay (20X)	1.25 µL
RNase P Assay (20X)	1.25 µL
RT-PCR Grade Water	11.25 µL
Total Reaction Mix volume	**20.0 µL**

2. For each reaction, combine the following components in a MicroAmp™ Optical 96-Well Reaction Plate (0.2-mL) well.

Component	Volume per well
Reaction Mix (see step 1)	20.0 µL
• Nucleic acid research sample *or* • 1 µL 2019-nCoV Control v1 + 4 µL RT-PCR Grade Water *or* • Negative control (NTC or NEC)	5.0 µL
Total reaction volume	**25.0 µL**

3. Set up and run the reactions on a real-time PCR instrument using the following settings:
   - Analysis method: Comparative C_t
   - Cycling mode: Standard

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Thermal Protocol

Note:

Only standard ramp rates were tested, even for fast plates on fast instruments

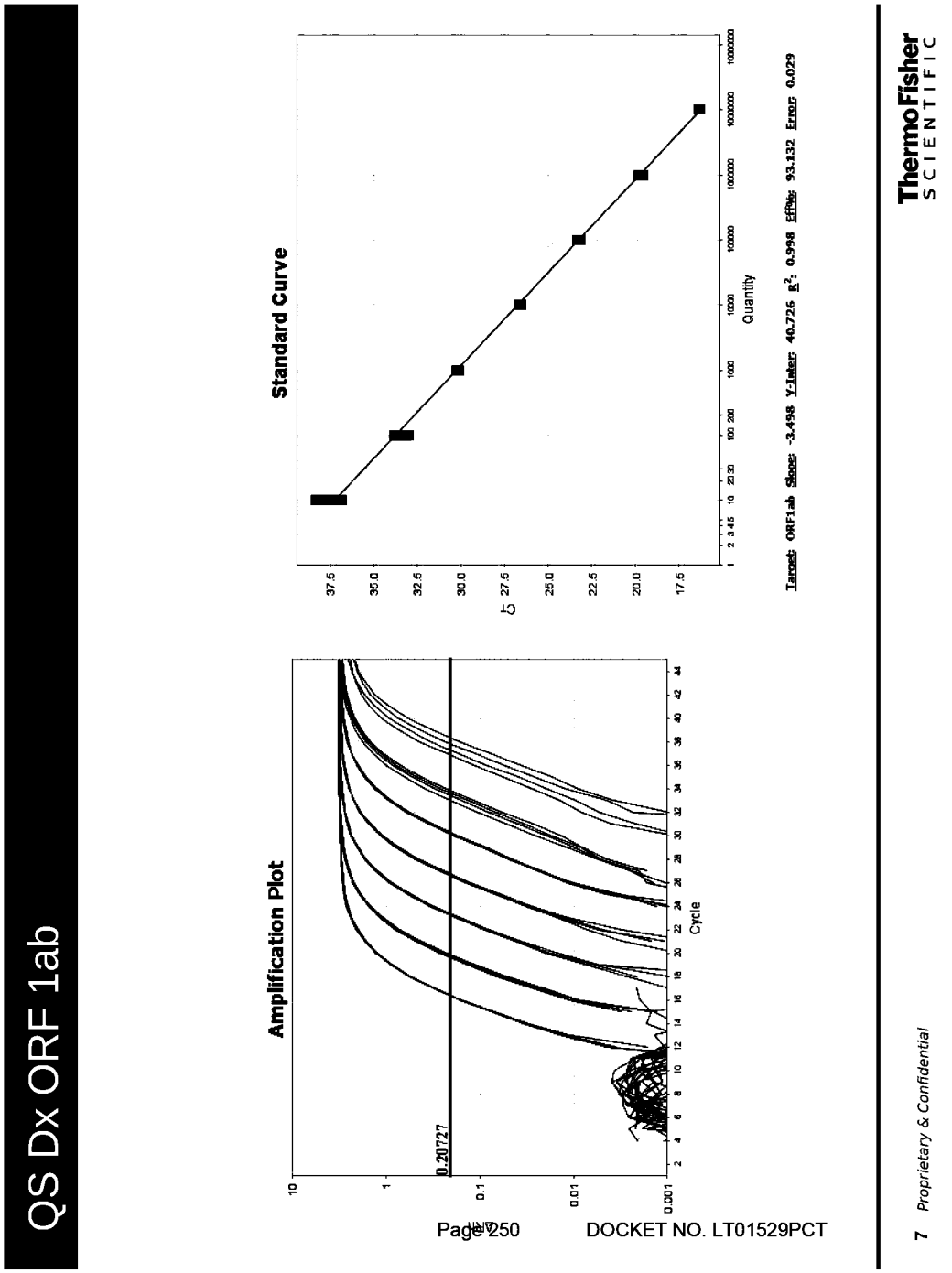
Stage	Step	Temperature	Time	
			TaqPath™ 1-Step RT-qPCR Master Mix, CG	TaqMan™ Fast Virus 1-Step Master Mix
Hold	UNG incubation[1]	25°C	2 minutes	N/A[2]
Hold	Reverse transcription	50°C	15 minutes	5 minutes
Hold	Activation[3]	95°C	2 minutes	20 seconds
Cycling (40 cycles)	Denaturation	95°C	3 seconds	3 seconds
	Anneal/Extension	60°C	30 seconds	30 seconds

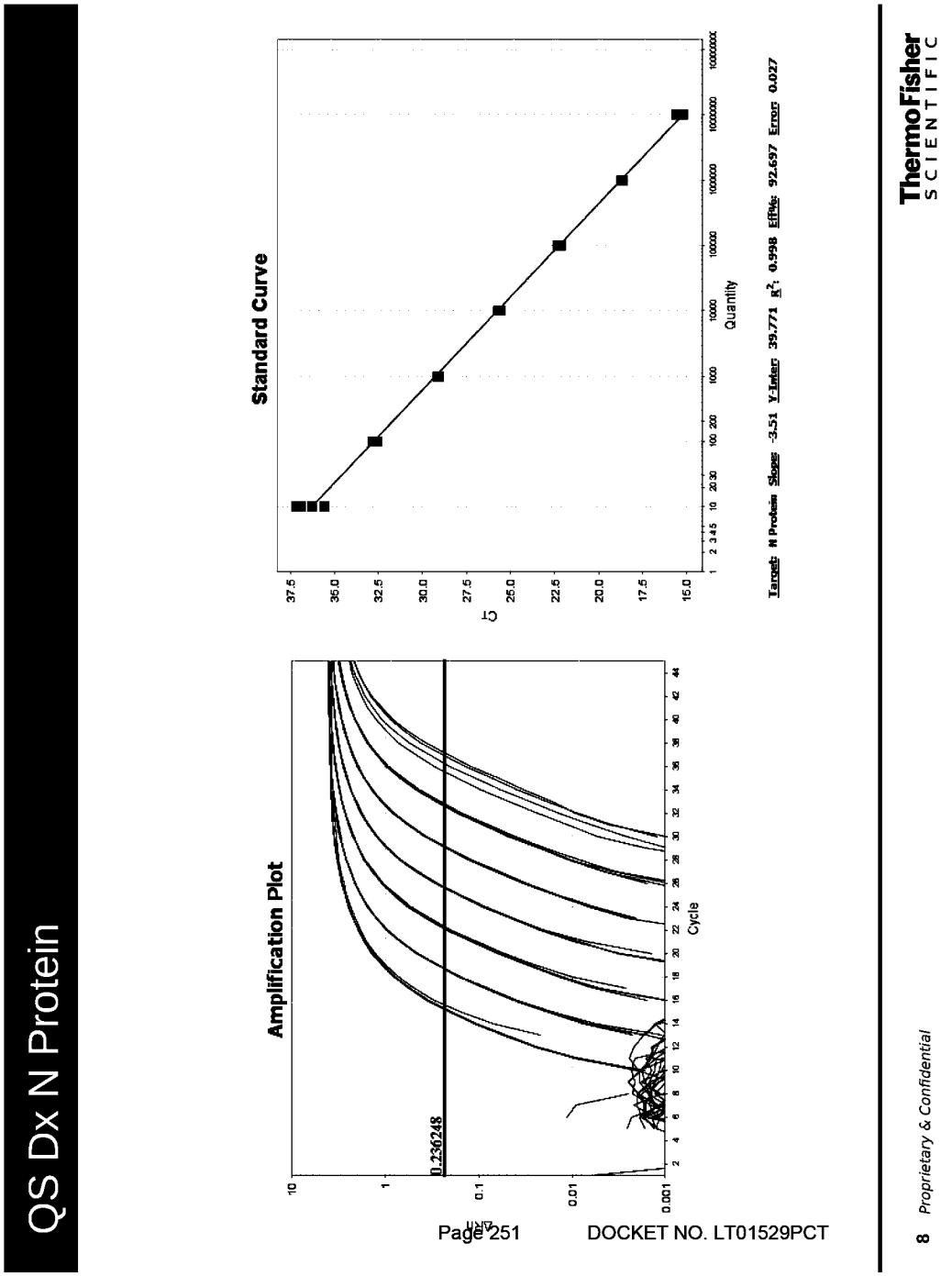
[1] Heat-labile UNG in TaqPath™ 1-Step RT-qPCR Master Mix, CG is completely inactivated during the first ramp to 95°C.
[2] TaqMan™ Fast Virus 1-Step Master Mix does not include UNG.
[3] Required for RT inactivation, first denaturation, and activation of the DNA polymerase.

Results shown are only from TaqMan Fast Virus 1-Step Master Mix.

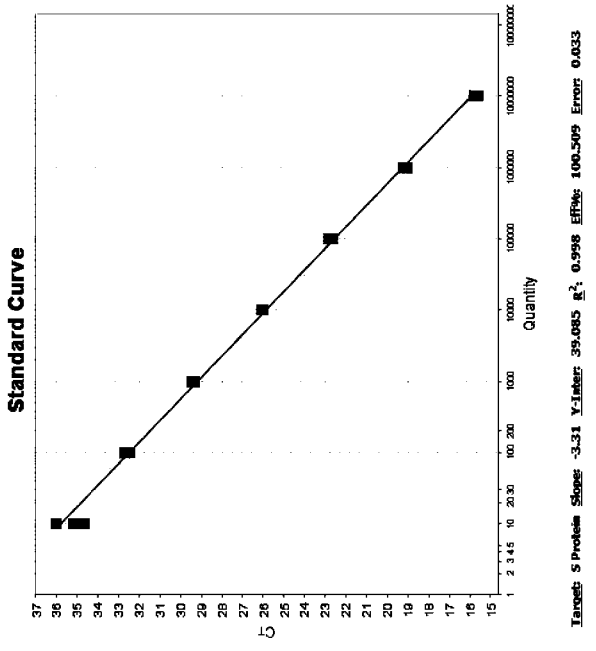
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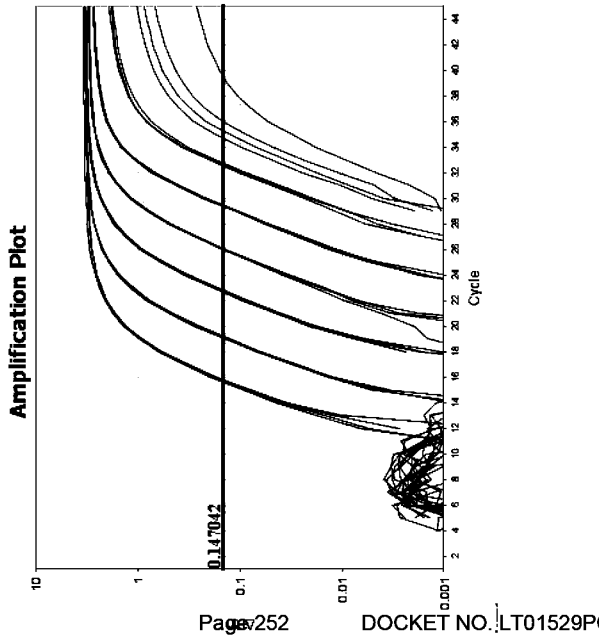
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QuantStudio™ Dx
Real-Time PCR Instrument
QS DX
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QS Dx ORF 1ab
Standard Curve
Quantity
Ct
Target: ORF1ab Slope: -3.498 Y-Inter: 40.726 R²: 0.998 Eff%: 93.132 Error: 0.029
Amplification Plot
Cycle
0.20727
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QS Dx N Protein
Standard Curve
Ct
Quantity
Target: N Protein Slope: -3.51 Y-Inter: 39.771 R²: 0.998 Eff%: 92.697 Error: 0.027
Amplification Plot
Cycle
0.236248
ΔRn
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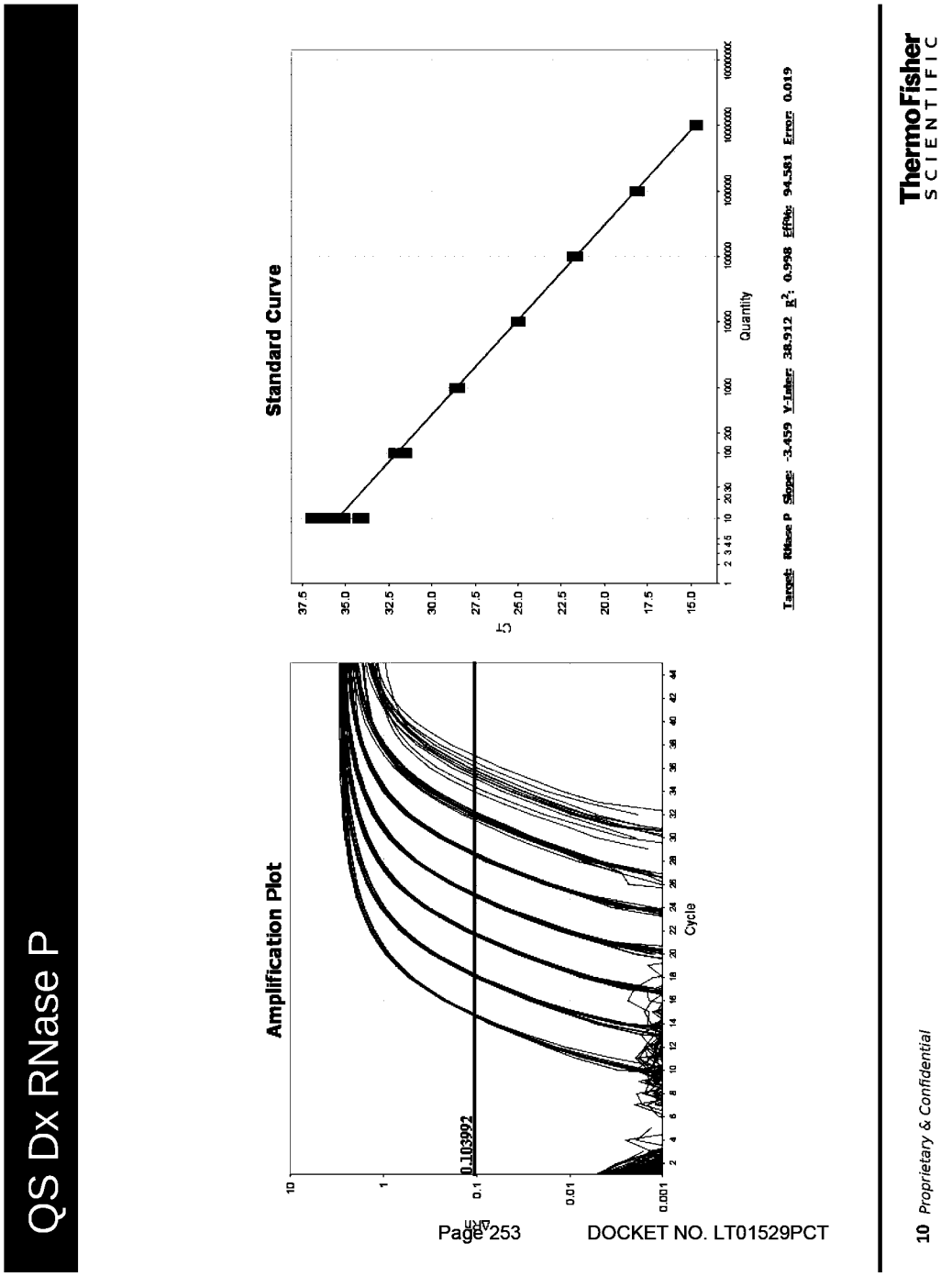
QS Dx S Protein





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QS Dx RNase P
Amplification Plot
Cycle
0.103992
Standard Curve
Quantity
Target: RNase P Slope: -3.459 Y-Inter: 38.912 R²: 0.998 Effic: 94.581 Error: 0.019
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QuantStudio 5 DX
Real-Time PCR System
QS 5 DX
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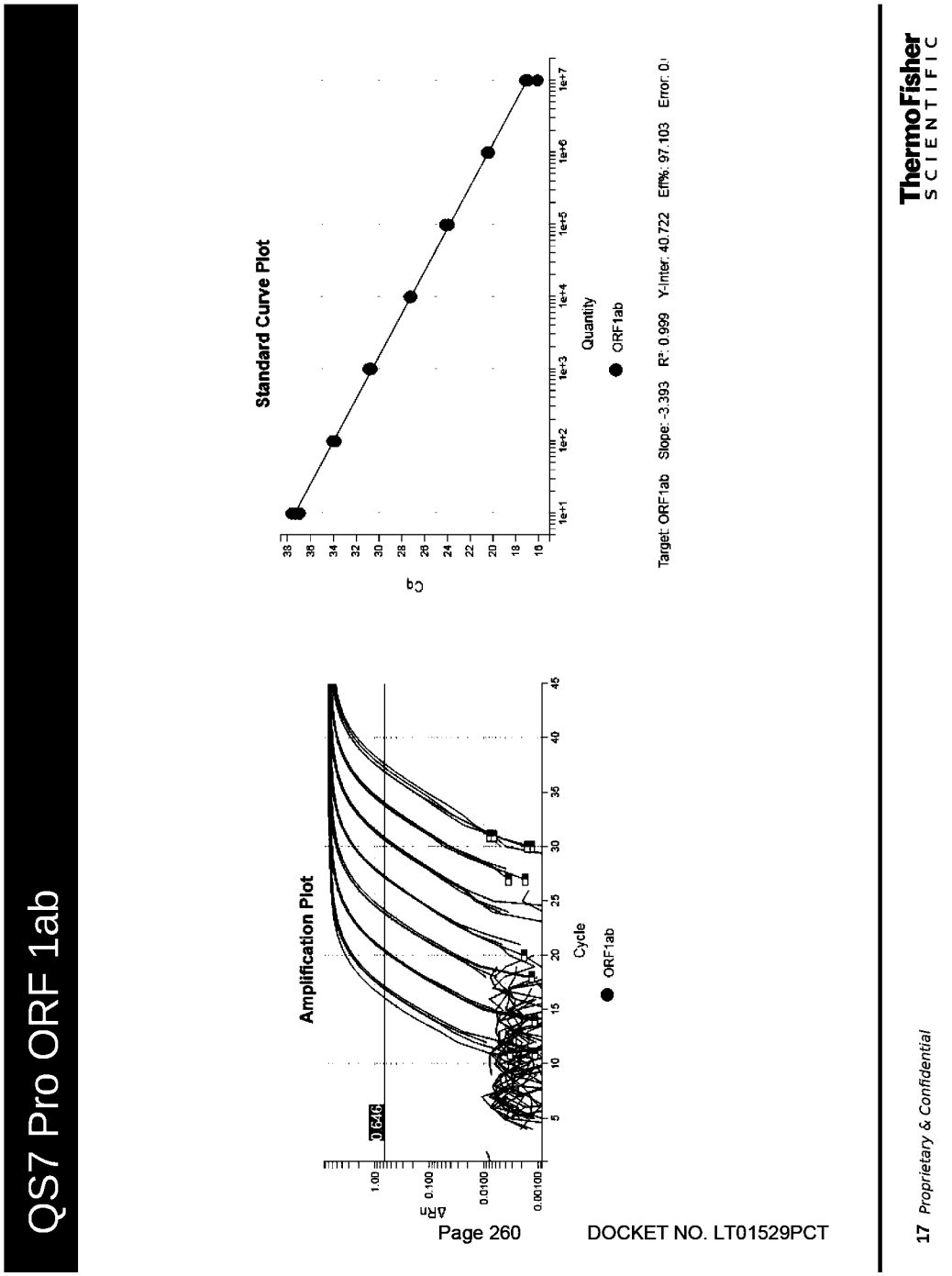
QS5 Dx ORF 1ab
Standard Curve
Quantity
Ct
N protein
orf1ab
S protein
RNase P
Amplification Plot
Cycle
Rn
ORF1ab
Page 255
DOCKET NO. LT01529PCT
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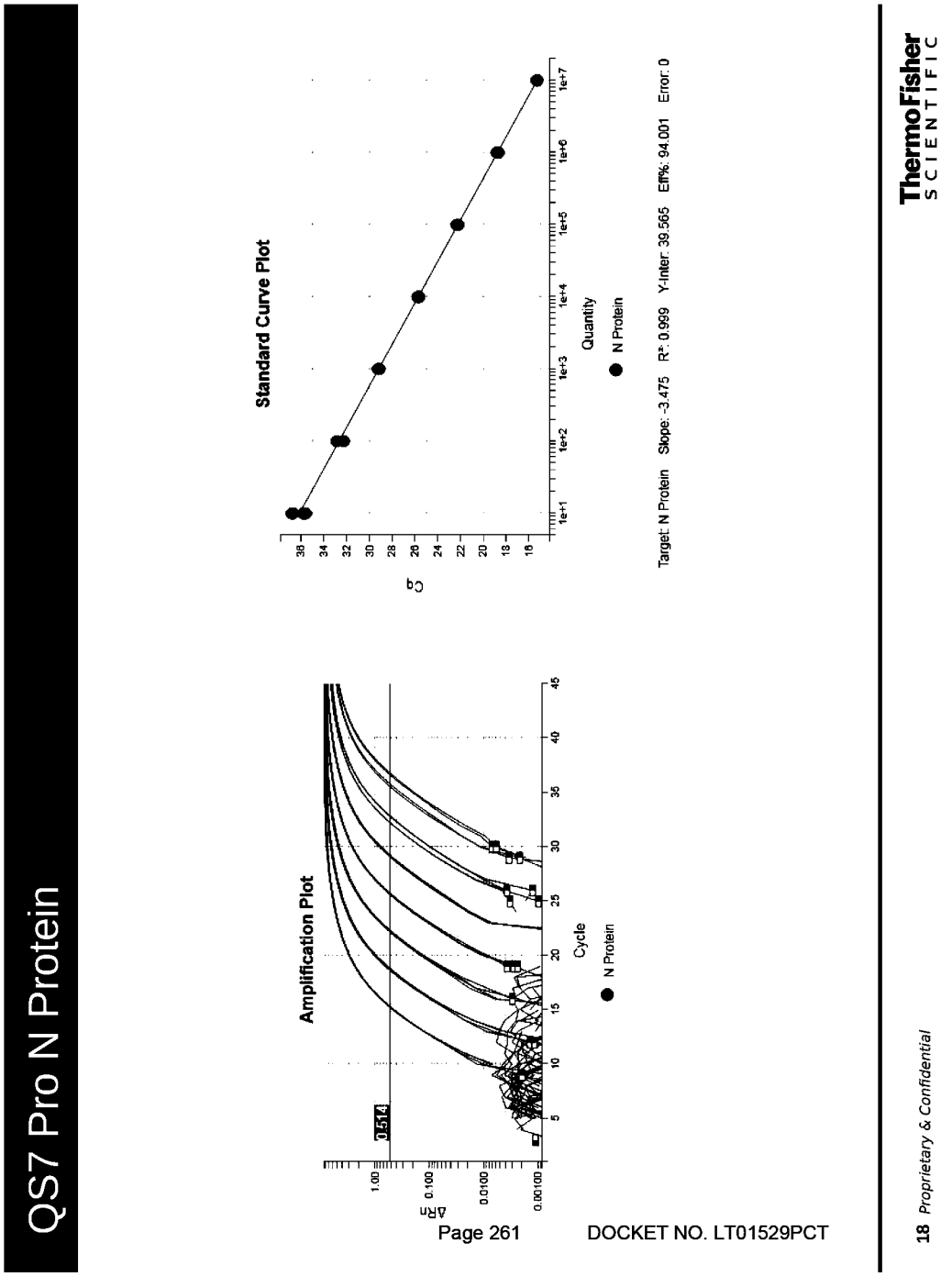
QS5 Dx N Protein
Standard Curve
Quantity
Ct
N protein
ORF1ab
S protein
RNase P
Amplification Plot
Cycle
Rn
N protein
ThermoFisher
SCIENTIFIC
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QS5 Dx S Protein
Standard Curve
Quantity
Ct
N protein
ORF1ab
S protein
RNase P
Amplification Plot
Cycle
Rn
0.145729
S protein
ThermoFisher
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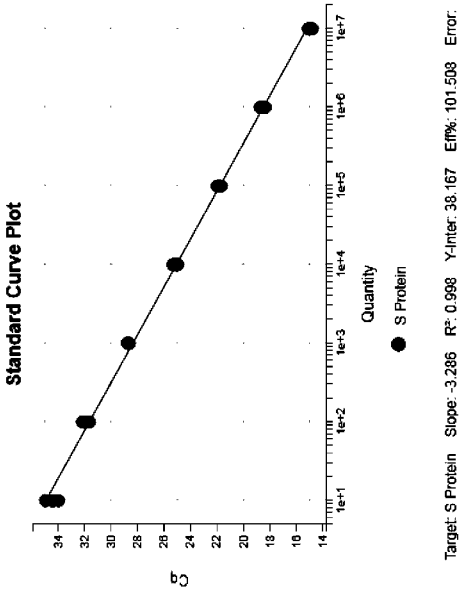
QS55 Dx RNase P
Standard Curve
Quantity
Ct
N protein
ORF1ab
S protein
RNase P
Amplification Plot
Cycle
Rn
0.137992
RNase P
ThermoFisher
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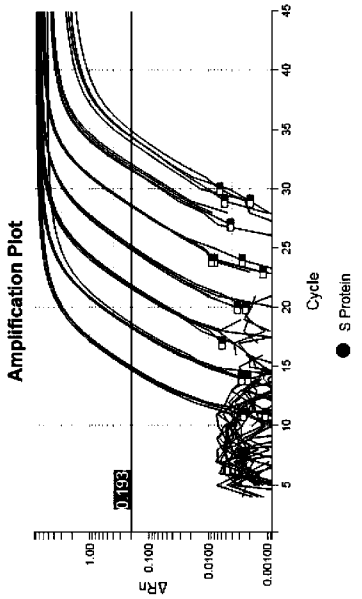
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QuantStudio 7 Pro
Real-Time PCR Systems
QS7 Pro
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QS7 Pro ORF 1ab
Standard Curve Plot
Quantity
ORF1ab
Target: ORF1ab Slope: -3.393 R²: 0.999 Y-Inter: 40.722 Eff%: 97.103
Amplification Plot
Cycle
ORF1ab
0.1646
ΔRn
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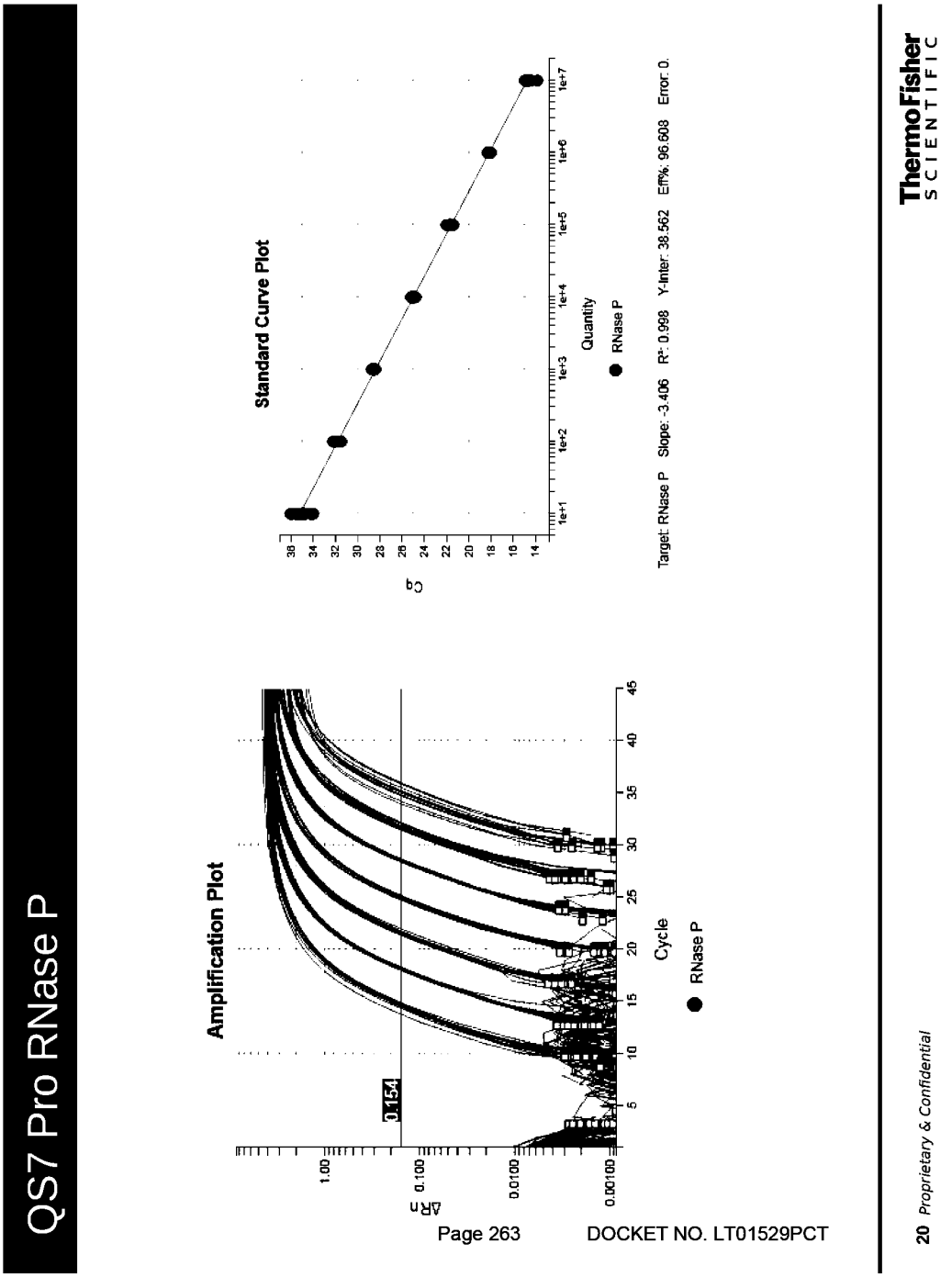

QS7 Pro N Protein
Standard Curve Plot
Quantity
N Protein
Target: N Protein Slope: -3.475 R²: 0.999 Y-Inter: 39.565 Eff%: 94.001 Error: 0
Amplification Plot
ΔRn
Cycle
N Protein
0.514
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QS7 Pro S Protein





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QS7 Pro RNase P
Amplification Plot
ΔRn
Cycle
RNase P
0.154
Standard Curve Plot
Ct
Quantity
RNase P
Target: RNase P Slope: -3.406 R²: 0.998 Y-Inter: 38.562 Eff%: 96.608 Error: 0.
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Mean Ct comparison at 10 copy inputs

Instrument	ORF1ab Mean Ct 10 copies	ORF1ab Ct SD 10 copies	N Protein Mean Ct 10 copies	N Protein Ct SD 10 copies	S Protein Mean Ct 10 copies	S Protein Ct SD 10 copies	RNase P Mean Ct 10 copies	RNase P Ct SD 10 copies
QS Dx	37.54	0.64	36.45	0.72	36.12	2.07	35.14	0.90
QS5 Dx	37.00	0.57	36.57	0.57	34.67	0.24	35.19	0.54
QS7 Pro	37.16	0.34	36.17	0.31	34.38	0.40	34.98	0.60

- Auto baseline / Auto threshold
- 4 replicates per instrument

Note:
- Results are from a 45-cycle run, not a 40-cycle run.
- Ct values will be higher if more cycles are added if curves do not reach a horizontal plateau at the end of the run.

Results interpretation

Expected results of the control reactions for each virus assay

Reaction	Expected Ct value	About unexpected results
NTC	Undetermined	If the NTC amplification curve crosses the threshold (false positive), sample contamination may have occurred. Repeat the test with new reagents.
2019-nCoV Control v1	Ct ≤ 37	If expected positive reactivity is not achieved, repeat the test with new reagents.

Individual assay result interpretation

Virus assay (FAM dye)	RNase P assay (IPC) (VIC dye)	Virus assay results
Ct ≤ 37	Any value	Positive
37 < Ct < 40	Any value	Inconclusive* Repeat the test.
Ct = Undetermined or Ct =40	Ct < 40	Negative**
Ct = Undetermined or Ct =40	Ct = Undetermined or Ct =40	Invalid Repeat the test

* Inconclusive: If the second run produces Ct < 37 or a value consistent with first run 37 ≤Ct < 40 the result is positive for the specific gene target.
** Negative: If MS2 (JUN channel) has no Ct value or Ct = 40, the run is invalid and must be repeated unless amplification is observed for other assays.

Note: If the viral target concentration is ≥ 10^5 copies/well, the MS2 Ct value may be higher than the MS2 Ct value of the positive control. This is the result of reagent competition and does not invalidate results.

Virus assay results interpretation

Virus assay results	Interpretation of results
Any two of the three assays are positive.	Virus RNA is present.
Any one of the assays is positive in two different samples collected from the same research subject.	Virus RNA is present.
All three of the assays are negative.	Virus RNA is not present.

APPENDIX 8

APPENDIX 8

The Critical Function of Mixing RT-PCR Reaction Plates for the SARS-CoV-2 assay

Purpose:

The purpose of this paper is to provide detailed instructions for adequate mixing of RT-PCR reaction plates and to provide supporting data demonstrating the value of these instructions.

Introduction:

To ensure proper analysis of SARS-CoV-2 research samples, it is essential to mix the RTPCR reaction properly by vortexing the plate, as mandated in the Instructions for Use. Failure to do so can result in Optical Mixing, a phenomenon that is likely to occur when the sample volume exceeds 20% of the PCR reaction volume. Optical Mixing can lead to RTPCR baseline instability, resulting in QC failure of entire plates and potential false classification of samples. An example of Optical Mixing leading to false classification of samples is presented in **Figure 1**.

Mixing Protocol:

1. After master mix, assay, water, samples, and controls have been added to the RT-PCR reaction plate, seal the plate well with a MicroAmp Optical Adhesive Film (4311971, 4360954). Use the MicroAmp Adhesive Film Applicator (4333183) to make sure all wells are sealed completely. The MicroAmp Optical Adhesive Cover uses a pressure-sensitive adhesive backing to adhere the cover to the optical 96- or 384-well plate. It is imperative that you use enough force to activate the pressure-sensitive adhesive to prevent evaporation from the wells.

2. Set the speed of a vortex mixer, such as the Vortex-Genie 2 from Scientific Industries (shown in **Figure 8**), to the highest setting and set mode to "Touch." Ensure that the vortex mixer has a platform rather than a tube cup installed.

3. Place the plate in contact with the vortex mixer and hold with medium pressure for 10 – 15 seconds. Ensure that the vortex platform is able to move vigorously and that the reaction mix moves freely in the wells; too much or too little pressure applied to the vortex platform will reduce mixing efficiency. Move the plate around during vortexing to ensure that contact with the platform has been made at all four quadrants and the center of the plate for equal time.

Experimental Design

In Experiment 1, two identical 96-well plates were created; one plate was vortexed for 30 seconds at maximum speed on a Vortex-Genie 2, and the other plate was not mixed at all. Each plate contained triplicate reactions of extracted contrived positive samples and negative samples. Contrived positive samples consisted of pooled nasopharyngeal specimens spiked with SARS-CoV-2 viral RNA at 9X, 3X or 1X the Limit of Detection (2,250 GCE/mL, 750 GCE/mL and 250 GCE/mL, respectively). Samples were extracted with either the MagMAX Viral/Pathogen Nucleic Acid Isolation Kit and a 400-µL specimen volume or the MagMAX Viral/Pathogen II Nucleic Acid Isolation Kit and a 200-µL specimen volume, and both extraction workflows were run on the same RT-PCR plate.

In Experiment 2, two identical 384-well plates were created; one plate was vortexed for 10 seconds at maximum speed on a Vortex-Genie 2, and the other plate was not mixed at all. Each plate contained 48 replicate reactions of extracted SARS-CoV-2 viral RNA at 10 GCE/reaction plus the MS2 Internal Control. RT-PCR runs were performed on an Applied Biosystems QuantStudio 7 Flex system with a 384-well block.

Results

As shown in **Figures 2**, **4** and **6**, the 96-well and 384-well plates that were not mixed demonstrated steep downward slopes in the fluorescent signal during the early cycles of the thermal protocol. By contrast, **Figures 3**, **5** and **7** reveal that proper mixing produces flatter baselines for the same conditions.

Conclusion

Compared with no mixing, vortexing for 10 – 30 seconds produces flatter baselines irrespective of extraction protocol, plate type or sample type. Because falling baselines can produce plate failures or inaccurate results, vortex mixing is vital for achieving reliable, specific results.

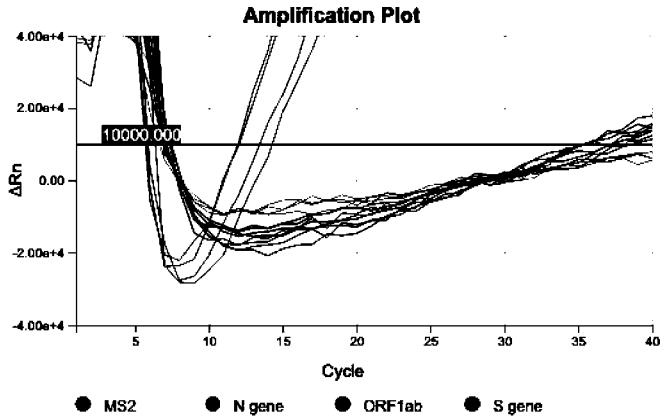


Figure 1. Example of Optical Mixing causing negative targets to cross the threshold. Confidential samples from a plate that was not mixed properly. In this case, the lack of mixing caused these four negative samples to be called Inconclusive.

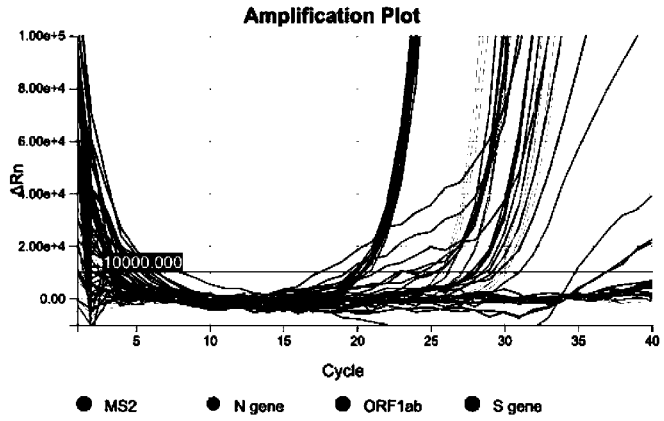


Figure 2. MagMAX Viral/Pathogen 400-µL protocol without mixing. Contrived positive samples at 9X, 3X and 1X LoD and negative samples were extracted in triplicate and used as templates for RT-PCR with no mixing.

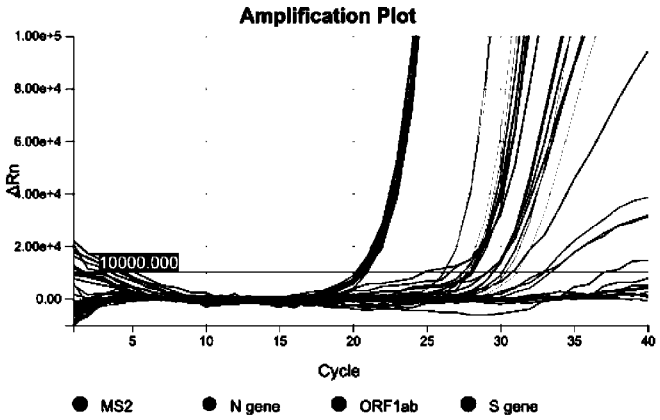


Figure 3. MagMAX Viral/Pathogen 400-µL protocol with vortex mixing.

Contrived positive samples at 9X, 3X and 1X LoD and negative samples were extracted in triplicate and used as templates for RT-PCR with 30 seconds of vortex mixing.

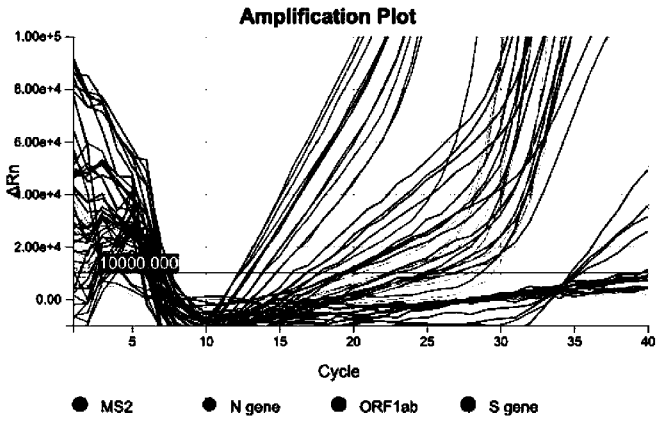


Figure 4. MagMAX Viral/Pathogen II 200-µL protocol without mixing.

Contrived positive samples at 9X, 3X and 1X LoD and negative samples were extracted in triplicate and used as templates for RT-PCR with no mixing.

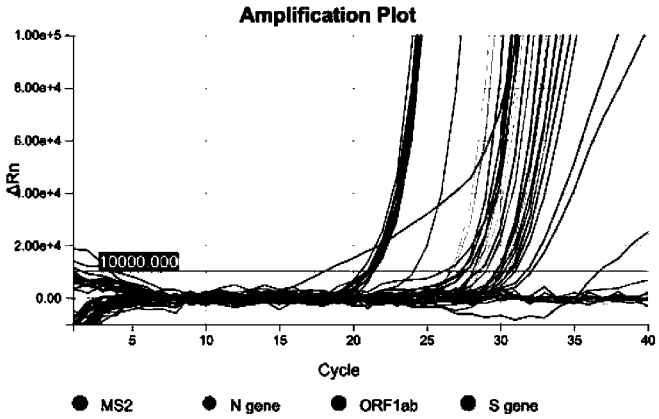


Figure 5. MagMAX Viral/Pathogen II 200-µL protocol with vortex mixing. Contrived positive samples at 9X, 3X and 1X LoD and negative samples were extracted in triplicate and used as templates for RT-PCR with 30 seconds of vortex mixing.

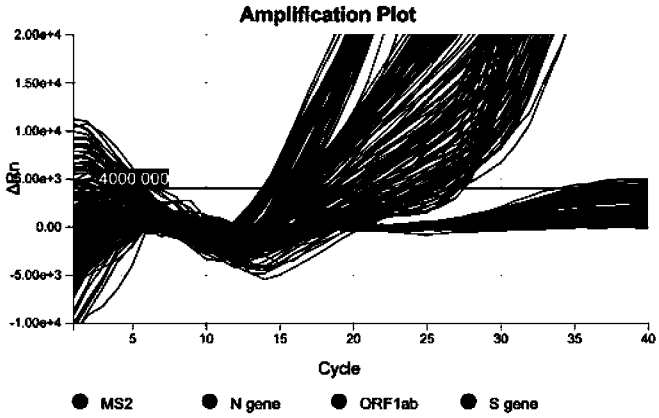


Figure 6. SARS CoV-2 viral RNA without mixing. Purified SARS-CoV-2 viral RNA at LoD was used as a template for RT-PCR in 48 replicate reactions without mixing. The run was performed on a QuantStudio 7 Flex system with a 384-well block.

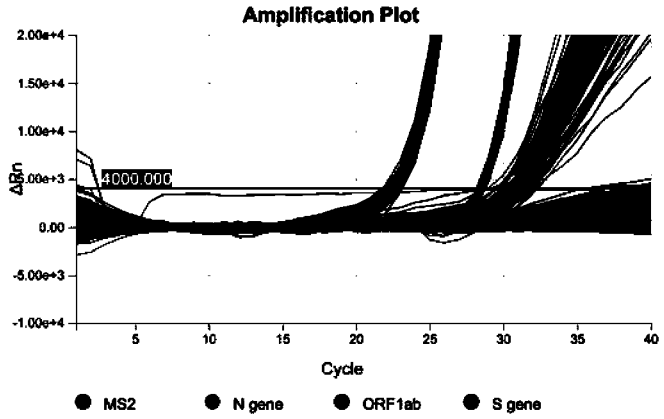


Figure 7. SARS CoV-2 viral RNA with vortex mixing. Purified SARS-CoV-2 viral RNA at LoD was used as a template for RT-PCR in 48 replicate reactions with 10 seconds of vortex mixing. The run was performed on a QuantStudio 7 Flex system with a 384-well block.

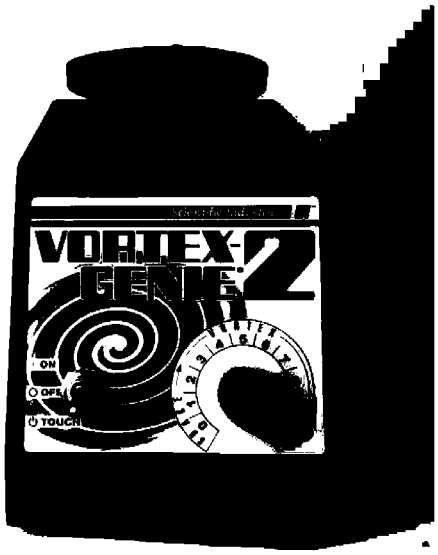


Figure 8. Photo of a Vortex-Genie 2 with recommended settings. This vortex model was used in the studies presented in this paper.

APPENDIX 9

appliedbiosystems QUICK REFERENCE

TaqMan™ SARS-CoV-2, Flu A/B, RSV Multiplex Assay

Pub. No. MAN0019614 Rev. A.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

The TaqMan™ SARS-CoV-2, Flu A/B, RSV Multiplex Assay is a multiplex real-time RT-PCR assay for the detection of RNA from the SARS-CoV-2 virus, influenza A and B viruses, and respiratory syncytial virus (RSV) subtypes A and B.

The assay requires the following components:

- TaqMan™ SARS-CoV-2, Flu A/B, RSV RT-PCR Assay Kit, includes the following components:
  - TaqMan™ SARS-CoV-2, Flu A/B, RSV Multiplex Assay—Multiplexed assays that contain primer and probe sets specific to the following targets (see Table 1 on page 1):
    - SARS-CoV-2 N protein and SARS-CoV-2 S protein
    - Flu A/B
    - RSV
    - MS2
  - TaqMan™ MS2 Phage Control—Internal process control for nucleic acid extraction
- TaqMan™ SARS-CoV-2, Flu A/B, RSV RNA Control—RNA control that contains targets specific to the SARS-CoV-2, influenza A and B, and RSV genomic regions targeted by the assays
- TaqMan™ Control Dilution Buffer—Dilution buffer for the control
- TaqPath™ 1-Step Multiplex Master Mix

Table 1 Dyes, quenchers, and targets

Dye	Quencher	Target
FAM™ dye	QSY™ quencher	Flu (A and B)
VIC™ dye	QSY™ quencher	SARS-CoV-2 (N and S protein)
ABY™ dye	QSY™ quencher	RSV
JUN™ dye	QSY™ quencher	MS2

Note: The viral targets are each in a single optical channel and cannot be differentiated.

For catalog numbers and storage conditions, see “Contents and storage” on page 2.

IMPORTANT! It is the responsibility of the laboratories using the TaqMan™ SARS-CoV-2, Flu A/B, RSV Multiplex Assay to design and validate their own experimental design and analysis parameters.

Contents and storage

The items listed in the following table are required for the TaqMan™ SARS-CoV-2, Flu A/B, RSV Multiplex Assay. The items listed are sufficient for 1,000 reactions.

Kit or product	Cat. No.	Components	Amount	Storage
TaqMan™ SARS-CoV-2, Flu A/B, RSV RT-PCR Assay Kit	A47702	TaqMan™ SARS-CoV-2, Flu A/B, RSV Multiplex Assay	1,500 µL	–30°C to –10°C
		TaqMan™ MS2 Phage Control	10 × 1 mL	–30°C to –10°C
TaqMan™ SARS-CoV-2, Flu A/B, RSV RNA Control	956126	—	10 × 10 µL	≤ –70°C
TaqMan™ Control Dilution Buffer	A49889	—	10 × 250 µL	–30°C to –10°C
TaqPath™ 1-Step Multiplex Master Mix	A28523	—	10 mL	–30°C to –10°C

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

IMPORTANT! The customer is responsible for performing all of the necessary validations to run this assay.

Item	Source
Real-time PCR instrument	
An Applied Biosystems™ real-time PCR instrument compatible with the four dyes listed in Table 1 on page 1. The assay has been tested with the following instrument: Applied Biosystems™ 7500 Fast Real-Time PCR Instrument (used with SDS Software v1.5.1 or 7500 Software v2.3)	Contact your local sales office
Software	
QuantStudio™ Design and Analysis Software v2.4.3 or later	thermofisher.com/qpcrsoftware
Equipment	
Laboratory freezers • –30°C to –10°C • ≤ –70°C	MLS
Centrifuge, with a rotor that accommodates standard and deepwell microplates	MLS
Microcentrifuge	MLS
Laboratory mixer, vortex or equivalent	MLS
Single and multichannel adjustable pipettors (1.00 µL to 1,000.0 µL)	MLS
Cold block (96-well or 384-well) or ice	MLS

Item	Source
Automated nucleic acid extraction system and materials	
KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head	5400630
KingFisher™ Flex 96 Deep-Well Heating Block	24075430
KingFisher™ Deep-Well 96 Plate	95040450, A48305, A48424, 95040455
96-well plate for the tip comb, one of the following: • KingFisher™ 96 KF microplate • Tip Comb Presenting Plate for KF 96 • Nunc™ MicroWell™ 96-Well Microplate, Flat Bottom • Nunc™ MicroWell™ 96-Well Microplate, barcoded • ABgene™ 96–Well Polypropylene Storage Microplate • ABgene™ 96–Well 1.2–mL Polypropylene Deepwell Storage Plate • Nunc™ F96 MicroWell™ Black Polystyrene Plate • Nunc™ F96 MicroWell™ White Polystyrene Plate • KingFisher™ Deep-Well 96 Plate	 • 97002540 • 267600 • 167008 • 269787 • AB0796 • AB1127 • 137101 • 136101 • 95040450, A48305, A48424, 95040455
KingFisher™ 96 tip comb for DW magnets	97002534, A48438, A48414
Kits and reagents	
MagMAX™ Viral/Pathogen II Nucleic Acid Isolation Kit	A48383
Fisher BioReagents™ Ethanol, Absolute, Molecular Biology Grade[1], or equivalent	BP2818100, BP2818500, BP28184
Nuclease-free Water (not DEPC-Treated)	MLS
Calibration plates (7500 Real-Time PCR Instrument series)	
ABY™ Dye Spectral Calibration Plate for Multiplex qPCR, Fast 96-well (0.1-mL)	A24734
JUN™ Dye Spectral Calibration Plate for Multiplex qPCR, Fast 96-well (0.1-mL)	A24735
Tubes, plates, and other consumables	
MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL	4346906, 4366932
MicroAmp™ Fast Optical 96-Well Reaction Plate, 0.1 mL	4346907
MicroAmp™ Clear Adhesive Film	4306311
MicroAmp™ Optical Adhesive Film	4311971, 4360954
MicroAmp™ Adhesive Film Applicator	4333183
Nonstick, RNase-free microcentrifuge tubes (1.5 mL and 2.0 mL)	thermofisher.com/plastics
Sterile aerosol barrier (filtered) pipette tips	thermofisher.com/pipettetips

[1] Available at fisherscientific.com.

General laboratory recommendations

- Implement standard operating procedures in your laboratory to prevent contamination, such as the following:
  - Frequent glove changes
  - Frequent decontamination of surfaces, equipment, and pipettes with 10% bleach or decontamination solution, followed by 70% ethanol
  - Use of ultraviolet light during biosafety cabinet decontamination (when available)
- To prevent degradation, keep eluted sample RNA, master mixes, assays, and controls on ice or in cold blocks while in use. Limit freeze-thaw cycles.
- Aliquot reagents to prevent stock contamination and reduce the number of freeze-thaw cycles.
- After each run, review the amplification curves in the instrument software according to data QC standard operating procedures for your lab.

Extract RNA

IMPORTANT! It is the responsibility of the laboratories to validate their own experimental design, including RNA extraction.

Before you begin

Note: During the wash steps, the Wash Solution may develop inert white or brown particulates that float in solution. This is not a cause for concern and does not negatively affect performance.

- Extract RNA from 400 µL of sample.
- Determine the number of required reactions based on the number of samples, plus one Negative Control per plate.
- Prepare fresh 80% Ethanol using Ethanol, Absolute, Molecular Biology Grade and Nuclease-free Water (not DEPC-Treated) for the required number of reactions, sufficient for 1 mL per reaction, plus 10% overage.
- Label the short side of each KingFisher™ Deep-Well 96 Plate (4):

Label	Number of plates
Sample plate	1
Wash 1	1
Wash 2	1
Elution plate	1

- Label the short side of the KingFisher™ 96 KF microplate (1):

Label	Number of plates
Tip comb	1

Note: The following items can be used to hold the tip comb instead of the KingFisher™ 96 KF microplate:

- Tip Comb Presenting Plate for KF 96
- Nunc™ MicroWell™ 96-Well Microplate, Flat Bottom
- Nunc™ MicroWell™ 96-Well Microplate, barcoded
- ABgene™ 96–Well Polypropylene Storage Microplate
- ABgene™ 96–Well 1.2–mL Polypropylene Deepwell Storage Plate
- Nunc™ F96 MicroWell™ Black Polystyrene Plate
- Nunc™ F96 MicroWell™ White Polystyrene Plate
- KingFisher™ Deep-Well 96 Plate

- Mark the Negative Control well on the plate.

Set up the instrument

1. Ensure that the KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head is set up with the KingFisher™ Flex 96 Deep-Well Heating Block.

   IMPORTANT! Failure to use the proper magnetic head and heat block results in lower yields and potential harm to the instrument.

2. Ensure that the **MVP_2Wash_400_Flex** program has been downloaded from the MagMAX™ Viral/Pathogen II Nucleic Acid Isolation Kit product page at **www.thermofisher.com** and loaded onto the instrument.

Prepare the processing plates

Prepare the processing plates according to the following table. Cover the plates with a temporary seal (such as MicroAmp™ Clear Adhesive Film), then store at room temperature for up to 1 hour while you set up the sample plate.

Plate ID	Plate position	Plate type	Reagent	Volume per well
Wash 1 Plate	2	KingFisher™ Deep-Well 96 Plate	Wash Solution	1,000 µL
Wash 2 Plate	3		80% Ethanol	1,000 µL
Elution Plate	4		Elution Solution	50 µL
Tip Comb Plate	5	Place a KingFisher™ 96 tip comb for DW magnets in a KingFisher™ 96 KF microplate		

Note: The following items can be used to hold the tip comb instead of the KingFisher™ 96 KF microplate:

- Tip Comb Presenting Plate for KF 96
- Nunc™ MicroWell™ 96-Well Microplate, Flat Bottom
- Nunc™ MicroWell™ 96-Well Microplate, barcoded
- ABgene™ 96–Well Polypropylene Storage Microplate
- ABgene™ 96–Well 1.2–mL Polypropylene Deepwell Storage Plate
- Nunc™ F96 MicroWell™ Black Polystyrene Plate
- Nunc™ F96 MicroWell™ White Polystyrene Plate
- KingFisher™ Deep-Well 96 Plate

Prepare Binding Bead Mix

Prepare the required amount of Binding Bead Mix on each day of use.

1. Vortex the Total Nucleic Acid Magnetic Beads to ensure that the bead mixture is homogeneous.
2. For the number of required reactions, prepare the Binding Bead Mix according to the following table:

Component	Volume per well[1]	Volume per 96-well plate
Binding Solution	530 µL	56.0 mL
Total Nucleic Acid Magnetic Beads	20 µL	2.1 mL
Total volume per well	**550 µL**	**58.1 mL**

[1] Include 10% overage when preparing the Binding Bead Mix for use with multiple reactions.

3. Mix well by inversion, then store at room temperature.

Prepare a Proteinase K and TaqMan™ MS2 Phage Control Mix

Prepare the required amount of the Proteinase K and TaqMan™ MS2 Phage Control Mix on each day of use. Keep on ice.

1. Thaw the vial of TaqMan™ MS2 Phage Control.
2. For the number of required reactions, prepare the Proteinase K and TaqMan™ MS2 Phage Control Mix according to the following table:

Component	Volume per well[1]	Volume per 96-well plate
Proteinase K	10 µL	1,056 µL
TaqMan™ MS2 Phage Control	10 µL	1,056 µL
Total volume per well	**20 µL**	**2,112 µL**

[1] Include 10% overage when preparing the Proteinase K and TaqMan™ MS2 Phage Control Mix for use with multiple reactions.

3. Mix well by inversion, then store on ice.

Prepare sample plate

Prepare the Proteinase K and TaqMan™ MS2 Phage Control Mix (see "Prepare a Proteinase K and TaqMan™ MS2 Phage Control Mix" on page 6).

1. Invert the Binding Bead Mix 5 times gently to mix, then add 550 µL to each sample well and the Negative Control well in the Sample Plate.

   Note: Remix the Binding Bead Mix by inversion frequently during pipetting to ensure even distribution of beads to all samples or wells. The Binding Bead Mix is viscous, so pipet slowly to ensure that the correct amount is added. DO NOT reuse pipette tips to add Binding Bead Mix to the samples, as the high viscosity will cause variations in the volumes added.

2. Add 400 µL of sample to each sample well.
3. Add 400 µL of Nuclease-free Water (not DEPC-Treated) to the Negative Control well.
4. Add 20 µL of the Proteinase K and TaqMan™ MS2 Phage Control Mix to each well in the KingFisher™ Deep-Well 96 Plate labeled "Sample Plate", including the Negative Control well.

Process the samples

1. Select the **MVP_2Wash_400_Flex** on the KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head.
2. Start the run, then load the prepared plates into position when prompted by the instrument.
3. After the run is complete (~24 minutes after start), immediately remove the Elution Plate from the instrument, then cover the plate with MicroAmp™ Clear Adhesive Film.

   IMPORTANT! To prevent evaporation, seal the plate containing the eluate immediately.

   The samples are eluted in 50 µL of Elution Solution (see "Prepare the processing plates" on page 5).

Note: Significant bead carry over may adversely impact RT-PCR performance.

Place the Elution Plate on ice for immediate use in real-time RT-PCR.

Prepare RT-PCR reactions

Guidelines for RT-PCR

IMPORTANT!

- Prepare the run plate on ice and keep it on ice until it is loaded into the real-time PCR instrument.
- Run the plate immediately after preparation. Failure to do so could result in degraded RNA samples.
- To prevent contamination, prepare reagents in a PCR workstation or equivalent amplicon-free area. Do not use the same pipette for controls and RNA samples, and always use aerosol barrier pipette tips.
- Maintain an RNase-free environment.
- Protect assays from light.
- Keep RNA samples and components on ice during use.
- For each RT-PCR plate, include the following controls:
  - One Positive Control
  - One Negative Control from each extraction run.

Prepare the RT-PCR reactions

1. If frozen, thaw the reagents on ice.
2. Gently vortex the reagents, then centrifuge briefly to collect liquid at the bottom of the tube.
3. Dilute TaqMan™ SARS-CoV-2, Flu A/B, RSV RNA Control to a working stock (1/3,500 dilution):
   a. Pipet 98.0 µL of TaqMan™ Control Dilution Buffer into a microcentrifuge tube, then add 2.0 µL of TaqMan™ SARS-CoV-2, Flu A/B, RSV RNA Control. Mix well, then centrifuge briefly.
   b. Pipet 138.0 µL of TaqMan™ Control Dilution Buffer into a second microcentrifuge tube, then add 2.0 µL of the dilution created in substep 3a. Mix well, then centrifuge briefly.

   Note: The TaqMan™ SARS-CoV-2, Flu A/B, RSV RNA Control does not contain the MS2 template.
4. Prepare the Reaction Mix:
   a. For each run, combine the following components sufficient for the number of RNA samples plus one Positive Control and one Negative Control.

   All volumes include 10% overage for pipette error.

Component	Volume per RNA Sample or Control	Volume for *n* RNA Samples plus 2 Controls	Volume for 94 RNA Samples plus 2 Controls
TaqPath™ 1-Step Multiplex Master Mix (No ROX™) (4X)	6.25 µL	6.875 x (*n* + 2) µL	660 µL
TaqMan™ SARS-CoV-2, Flu A/B, RSV Multiplex Assay	1.25 µL	1.375 x (*n* + 2) µL	132 µL
Total Reaction Mix volume	**7.5 µL**	—	**792 µL**

5. Set up the reaction plate:
   a. Pipette 7.5 µL of the Reaction Mix prepared in step 4 into each well of a MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL.

      Plates without a barcode can be used (see "Required materials not supplied" on page 2).
   b. Gently vortex the sealed plate containing the purified sample RNA and Negative Control from the RNA extraction procedure, then centrifuge briefly to collect liquid at the bottom of the plate.
   c. Unseal the plate containing the purified sample RNA and Negative Control from the RNA extraction procedure. Add either sample RNA, Negative Control, or Positive Control to each well of the reaction plate according to Table 2 on page 8.

d. Seal the plate thoroughly with MicroAmp™ Optical Adhesive Film.

IMPORTANT! When applying the MicroAmp™ Optical Adhesive Film, ensure that pressure is applied across the entire plate and that there is a tight seal across every individual well. Failure to do so runs the risk of an improperly sealed well, leading to potential well-to-well contamination during vortexing and PCR.

e. Vortex the plate at the highest setting speed for 10–30 seconds with medium pressure. Move the plate around to ensure equal contact on the vortex mixer platform.

IMPORTANT! Vortex for 10–30 seconds to ensure proper mixing. Failure to do so might result in inaccurate sample results.

f. Centrifuge the reaction plate for 1–2 minutes at ≥650 × *g* (≥650 RCF) to remove bubbles and to collect the liquid at the bottom of the reaction plate.

Table 2 Reaction plate

Component	Volume per reaction		
	RNA Sample reaction	**Positive Control reaction**	**Negative Control reaction**
Reaction Mix (from step 4)	7.5 µL	7.5 µL	7.5 µL
Purified sample RNA (from RNA extraction)	17.5 µL	—	—
Positive Control (diluted TaqMan™ SARS-CoV-2, Flu A/B, RSV RNA Control from step 3)	—	17.5 µL	—
Negative Control (from RNA extraction)	—	—	17.5 µL
Total volume	**25.0 µL**	**25.0 µL**	**25.0 µL**

Set up and run the real-time PCR instrument

Calibration

Ensure that your real-time PCR instrument is calibrated for the dyes listed in Table 1 on page 1. See your instrument user guide for more information.

Dye calibration for the 7500 Real-Time PCR Instrument series

A maintained instrument will be calibrated for many dyes, including FAM™ dye and VIC™ dye. In addition to those dyes, the instrument operator must calibrate the instrument for ABY™ dye and JUN™ dye that are used with this kit. For all other assays, refer to the standard calibration process.

Peform RT-PCR

For more information about the instrument, see “Related documentation” on page 9.

1. Set up and run the real-time PCR instrument with the following settings.
   - Assay: Standard curve
   - Run mode: Standard
   - Passive reference set to **None**
   - Sample volume: 25 µL

2. Set up the following reporter dye and detector pairs.

Reporter dye	Detector
FAM	Flu (A and B)
VIC	SARS-CoV-2 (N and S protein)
ABY	RSV
JUN	MS2

3. Set up the thermal protocol.

Step	Temperature	Time	Number of cycles
UNG incubation	25°C	2 minutes	1
Reverse transcription	53°C	10 minutes	1
Preincubation	85°C	10 minutes	1
Activation	95°C	2 minutes	1
Denaturation	95°C	3 seconds	46
Anneal / extension	60°C	30 seconds	

4. Load the plate and start the instrument run.

Analyze data

IMPORTANT! It is the responsibility of the laboratories using the TaqMan™ SARS-CoV-2, Flu A/B, RSV Multiplex Assay to design and validate their own experimental design and analysis parameters.

For more information about using the software, see "Related documentation" on page 9.

Note: QuantStudio™ Design and Analysis Software v2 reports C_q values instead C_t values. The C_q values are equivalent to the C_t values indicated for data analysis and interpretation.

Use QuantStudio™ Design and Analysis Software v2.4.3 or later.

1. Open the data file (SDS) in the data analysis software.

   Note: QuantStudio™ Design and Analysis Software v2 requires data files created on a 7500 Fast Real-Time PCR Instrument to be saved as a new data file. Click **Actions ▸ Save As**, then save the data file with a new name.

2. Use automatic baselining with a start cycle of 5.

3. Set the appropriate threshold values for each target, as validated by your laboratory.

   IMPORTANT! Do not use automatic threshold values.

4. Determine C_t/C_q cutoff values for each target for samples and controls.

5. Analyze results according to analysis, interpretation, and QC parameters, as validated by your laboratory.

   IMPORTANT! After each run, review the data in the QuantStudio™ Design and Analysis Software v2.4.3 or later with the **Multicomponent Plot** view. This can identify amplification curves with inconsistencies that lead to inaccurate results.

Contact Support for more information.

Related documentation

Document	Publication Number
Applied Biosystems™ 7500/7500 Fast Real-Time PCR System: Maintenance Guide	4387777
MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (automated extraction) User Guide	MAN0018073
Thermo Scientific™ KingFisher™ Flex User Manual	N07669
QuantStudio™ Design and Analysis Software v2 User Guide	MAN0018200

Limited product warranty

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

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Revision history: Pub. No. MAN0019614

Revision	Date	Description
A.0	13 October 2020	New document.

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APPENDIX 10

applied biosystems QUICK REFERENCE

TaqCheck™ SARS-CoV-2 Fast PCR Assay

Catalog Numbers A47693

Pub. No. MAN0019744 Rev. A.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from thermofisher.com/support.

Product description

The TaqCheck™ SARS-CoV-2 Fast PCR Assay is a multiplex real-time RT-PCR assay for the detection of SARS-CoV-2 viral RNA in human saliva samples. The assay contains primer and probe sets specific to the following targets:

Table 1 Assay targets, dyes, and quenchers

Target	Dye	Quencher
SARS-CoV-2 N gene	VIC™ dye	QSY™ quencher
SARS-CoV-2 S gene		
Human RNase P RPP30 gene[1]	FAM™ dye	QSY™ quencher

[1] Serves as an internal positive control to monitor sample quality.

The assay requires the following components:

- TaqCheck™ SARS-CoV-2 Control—RNA control that contains SARS-CoV-2 N protein and S protein target regions
- TaqCheck™ SARS-CoV-2 Control Dilution Buffer—Dilution buffer for the control
- TaqPath™ 1-Step RT-qPCR Master Mix, CG

For catalog numbers and storage conditions, see "Contents and storage" on page 1.

IMPORTANT! It is the responsibility of the laboratories using the TaqCheck™ SARS-CoV-2 Fast PCR Assay to design and validate their own experimental design and analysis parameters.

Contents and storage

The items listed in the following table are required for the TaqCheck™ SARS-CoV-2 Fast PCR Assay. The items listed are sufficient for 1,200 reactions.

Kit or product	Cat. No.	Amount	Storage
TaqCheck™ SARS-CoV-2 Fast PCR Assay	A47693	690 µL	–30°C to –10°C
TaqCheck™ SARS-CoV-2 Control	956127	3 x 10 µL	≤ –70°C
TaqCheck™ SARS-CoV-2 Control Dilution Buffer	A50486	3 x 250 µL	–30°C to –10°C
TaqPath™ 1-Step RT-qPCR Master Mix, CG	• A15299 • A15300	• 5 x 1 mL • 1 x 10 µL	–30°C to –10°C

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

IMPORTANT! The customer is responsible for performing all of the necessary validations to run this assay.

Item	Source
Real-time PCR instrument and software	
An Applied Biosystems™ real-time PCR instrument compatible with the dyes listed in Table 1 on page 1. The assay was tested with the following instrument: Applied Biosystems™ QuantStudio™ 5 Real-Time PCR Instrument, 384-well block	Contact your local sales office
(*Recommended*) QuantStudio™ Design and Analysis Software v2.5 or later[1]	thermofisher.com/qpcrsoftware
Equipment	
Laboratory freezers • –30°C to –10°C • ≤ –70°C	MLS
BSL-2 biological safety cabinet	MLS
Centrifuge (capable of achieving 1,400 × g)	MLS
Microcentrifuge	MLS
Laboratory mixer, vortex or equivalent	MLS
Single and multichannel adjustable pipettors (2.00 µL to 1,000.0 µL)	MLS
Cold block (384-well) or ice	MLS
Heat block or water bath (capable of reaching 95°C)	MLS
Kits and reagents	
TBE Buffer (Tris-borate-EDTA) (10X),	B52, or equivalent
Tween®-20 Surfact-Amps™ Detergent Solution	28320
Nuclease-free Water (not DEPC-Treated)	MLS
70% Isopropanol spray or wipes	MLS
Tubes, plates, and other consumables	
Reservoir for multichannel pipettes	MLS
96-well plate	AB0796, or equivalent
MicroAmp™ Optical 384-Well Reaction Plate	• 4309849 (with barcode) • 4343370 (without barcode)
MicroAmp™ Clear Adhesive Film	4306311
MicroAmp™ Optical Adhesive Film	4311971, 4360954
MicroAmp™ Adhesive Film Applicator	4333183
Nonstick, RNase-free microcentrifuge tubes (1.5 mL and 2.0 mL)	thermofisher.com/microtubes
Sterile aerosol barrier (filtered) pipette tips	thermofisher.com/pipettetips
DNase and RNase-free tubes for mixing reagents (capable of mixing 5 mL and 50 mL)	MLS

[1] Use of QuantStudio™ Design and Analysis Software v2.5 is recommended, but not required. It is the responsibility of the laboratories using the assay to design and validate their own experimental design and analysis parameters.

General laboratory recommendations

- Implement standard operating procedures in your laboratory to prevent contamination, such as the following:
  - Frequent glove changes
  - Frequent decontamination of surfaces, equipment, and pipettes with 10% bleach or decontamination solution, followed by 70% ethanol
  - Use of ultraviolet light during biosafety cabinet decontamination (when available)
- Saliva samples should always be treated as if infectious and/or biohazardous in accordance with safe laboratory procedures.
- To prevent degradation, keep master mixes, assays, and controls on ice or in cold blocks while in use. Limit freeze-thaw cycles.
- Aliquot reagents to prevent stock contamination and reduce the number of freeze-thaw cycles.
- To ensure reliable performance of the real-time PCR instrument, perform preventive maintenance according to the instructions provided by the manufacturer in the instrument documentation (see "Related documentation" on page 7).

Guidelines for sample collection and storage

- Collect saliva sample in a sterile collection device with a leak-proof, screw-top lid.

  IMPORTANT! Do not collect saliva using a device that contains preservative solution.

- Collect a minimum of 1 mL saliva.
- Collect saliva samples according to the instructions provided with your collection device. We recommend that you follow best practices to minimize the presence of inhibitors in the saliva:
  - At least 30 minutes before collection, clean the mouth. Swish water for 10 seconds, then swallow to remove debris.
  - After cleaning the mouth, avoid eating, drinking, smoking, using chewing tobacco, chewing gum, brushing teeth, and using mouthwash or other foreign substances until the sample is collected.
  - During collection, allow saliva to passively pool in the mouth, then drool into the collection device. Do not cough while performing collection, and ensure that the sample is free of phlegm or other debris.

  Note: Laboratories are responsible for validation of their sample collection procedure.
- Store raw saliva samples according to the procedure established by your laboratory. For long-term storage, freeze raw saliva samples at -80°C. Avoid multiple freeze-thaw cycles.

Prepare saliva samples



WARNING! Saliva samples have the potential to transmit infectious diseases. Use safe laboratory procedures, including wearing personal protective equipment (PPE) and handling samples in a BSL-2 biological safety cabinet.

IMPORTANT! Saliva samples can contain high amounts of inhibitory compounds that can affect real-time RT-PCR results. Laboratories are responsible for validating their sample collection and preparation procedures for use with the assay.

Before you begin

- If the raw saliva samples are frozen, thaw completely at room temperature before processing.
- Ensure that the heating block or water bath is at 95°C.

Prepare 96-well plates with TBE Buffer-Tween®-20 Detergent (TBE-T) mix

1. For the required number of samples, prepare the TBE-T mix in a DNase and RNase-free tube, according to the following table:

Component	Volume per well	Volume per 96-well plate[1]	Volume per four 96-well plates[1]
TBE Buffer (10X)[2]	20 µL	2.4 mL	9.6 mL
Tween®-20 Detergent (10%)[3]	10 µL	1.2 mL	4.8 mL
Nuclease-free Water	70 µL	8.4 mL	33.6 mL
Total volume	**100 µL**	**12.0 mL**	**48.0 mL**

[1] Includes 25% overage.
[2] The TBE Buffer has a final concentration of 2X in the TBE-T mix.
[3] The Tween®-20 Detergent has a final concentration of 1% in the TBE-T mix.

2. Cap the tube, then mix well by inversion 5–10 times; do not vortex. Once mixed, allow bubbles to dissipate naturally.
3. For the required number of samples, add 100 µL of TBE-T mix to each well of a 96-well plate. Store the plates on ice.

Prepare the samples

Keep the saliva samples in the original tubes for the incubation step.

1. Incubate the saliva sample tubes in a water bath or heat block at 95°C for 30 minutes.
2. Remove the tubes from the water bath or heat block, then allow the samples to equilibrate to room temperature.
3. Vortex each sample at maximum speed for a minimum of 10 seconds, or until the sample appears homogenous.

   Note: Samples that are particularly viscous or contain high amounts of particulate may require longer vortex times. Some samples may contain particulate that does not fully homogenize.
4. Transfer 100 µL of each heat-treated saliva sample to the designated wells in the prepared TBE-T 96-well plates. Gently pipet up and down 10 times to mix. Ensure that you do not generate bubbles while you pipet.

Store the prepared sample plates on ice or at 4°C for up to 2 hours while setting up the RT-PCR.

Prepare RT-PCR reactions

Guidelines for RT-PCR

IMPORTANT!

- Prepare the RT-PCR plate on ice or a cold block. Keep the RT-PCR plate on ice or a cold block until it is loaded into the real-time PCR instrument.
- Run the RT-PCR plate within an hour after preparation. Failure to do so could result in degraded samples.
- To prevent contamination, prepare reagents in a PCR workstation or equivalent amplicon-free area. Do not use the same pipette for controls and samples, and always use aerosol barrier pipette tips.
- Maintain an RNase-free environment.
- Protect assays from light.
- Keep samples and components on ice or a cold block during use.
- For each RT-PCR plate, include the following controls:
  - One Positive Control
  - One No Template Control

Prepare the RT-PCR reactions

1. If frozen, thaw the reagents on ice or on a cold block.
2. Gently vortex the reagents, then briefly centrifuge the tube or swirl the bottle to collect the liquid at the bottom of the container.

3. Dilute TaqCheck™ SARS-CoV-2 Control to a working stock:
   a. Pipet 95.0 µL of TaqCheck™ SARS-CoV-2 Control Dilution Buffer into a microcentrifuge tube, then add 5.0 µL of TaqCheck™ SARS-CoV-2 Control. Mix well, then centrifuge briefly.
   b. Pipet 95.0 µL of TaqCheck™ SARS-CoV-2 Control Dilution Buffer into a second microcentrifuge tube, then add 5.0 µL of the dilution created in substep 3a. Mix well, then centrifuge briefly.

4. Prepare the Reaction Mix:
   a. For each 384-well plate, combine the following components sufficient for the number of RNA samples plus one Positive Control and one No Template Control.

Component	Volume per sample or control	Volume for *n* samples plus 2 controls[1]	Volume for 382 samples plus 2 controls[1]
TaqPath™ 1-Step RT-qPCR Master Mix, CG (4X)	2.5 µL	2.75 x (*n* + 2) µL	1,056.0 µL
TaqCheck™ SARS-CoV-2 Fast PCR Assay	0.5 µL	0.55 x (*n* + 2) µL	211.2 µL
Nuclease-free Water	2.0 µL	2.2 x (*n* + 2) µL	844.8 µL
Total Reaction Mix volume	**5.0 µL**	**—**	**2,112.0 µL**

[1] All volumes include 10% overage for pipette error.

5. Set up the reaction plate, according to the following table:

Component	Volume per reaction		
	Sample reaction	**Positive Control reaction**	**No Template Control reaction**
Reaction Mix (from step 4)	5.0 µL	5.0 µL	5.0 µL
Prepared sample (saliva + TBE-T)	5.0 µL	—	—
Positive Control (diluted TaqCheck™ SARS-CoV-2 Control from step 3)	—	2.0 µL	—
Nuclease-free Water	—	3.0 µL	5.0 µL
Total volume	**10.0 µL**	**10.0 µL**	**10.0 µL**

   a. Add 5.0 µL of the Reaction Mix prepared in step 4 to each well of a MicroAmp™ Optical 384-Well Reaction Plate.
   b. Add 5.0 µL of prepared sample (saliva plus TBE-T) to each sample well of the reaction plate.
   c. Add 2.0 µL of the diluted TaqCheck™ SARS-CoV-2 Control and 3.0 µL Nuclease-free Water to the Positive Control well of the reaction plate.
   d. Add 5.0 µL of Nuclease-free Water to the No Template Control well of the reaction plate.
   e. Seal the plate thoroughly with MicroAmp™ Optical Adhesive Film.

   IMPORTANT! When applying the MicroAmp™ Optical Adhesive Film, ensure that pressure is applied across the entire plate and that there is a tight seal across every individual well. Failure to do so runs the risk of an improperly sealed well, leading to potential well-to-well contamination during vortexing and PCR.

6. Vortex the reaction plate at the highest setting speed for 10–30 seconds with medium pressure. Move the plate around to ensure equal contact on the vortex mixer platform.

   IMPORTANT! Failure to vortex the plate for the recommended time can result in inaccurate sample results.

7. Centrifuge the reaction plate for 1–2 minutes at ≥1,400 × *g* (≥1,400 RCF) to remove bubbles and to collect the liquid at the bottom of the reaction plate.

Set up and run the real-time PCR

A maintained QuantStudio™ 5 Real-Time PCR System will be calibrated for FAM™ and VIC™ dyes. If calibration is required, refer to the standard calibration procedure in the instrument user guide.

1. Set up the real-time PCR instrument with the following settings.
   - Analysis type: Standard curve
   - Run mode: Fast
   - Passive reference: ROX
   - Sample volume: 10 µL
2. Set up the following reporter dye and detector pairs.

Reporter dye	Detector
FAM	RNAse P
VIC	SARS-CoV-2 N gene and SARS-CoV-2 S gene

3. Set up the thermal protocol.

Step	Temperature	Ramp rate	Time	Number of cycles
Reverse transcription	50°C	2.2°C per second	4 minutes	1
Activation	95°C	2.2°C per second	2 minutes	1
Denaturation	95°C	2.2°C per second	1 second	40
Anneal / extension	60°C	1.8°C per second	20 seconds	

4. Load the plate and start the instrument run.

Analyze data

IMPORTANT! It is the responsibility of the laboratories using the TaqCheck™ SARS-CoV-2 Fast PCR Assay to design and validate their own experimental design and analysis parameters.

(*Recommended*) Use QuantStudio™ Design and Analysis Software v2.5 or later. For more information about using the software, see "Related documentation" on page 7.

1. In the QuantStudio™ Design and Analysis Software v2 home screen, open the data file (EDS).
2. In the open data file, click **Actions ▸ Save As**, the save the data file with a new name.

   Note: QuantStudio™ Design and Analysis Software v2 requires data files created on a QuantStudio™ 5 Real-Time PCR System to be saved as a new data file.
3. In the analysis settings, select automatic baseline with a start cycle of 5.
4. Set the appropriate threshold values for each target, as validated by your laboratory.

   IMPORTANT! Do not use automatic threshold values.
5. Determine C_q cutoff values for each target for samples and controls.

   Note: QuantStudio™ Design and Analysis Software v2 reports C_q values instead C_t values. The C_q values are equivalent to C_t values.
6. Analyze results according to analysis, interpretation, and QC parameters, as validated by your laboratory.

Contact Support for more information.

DOCKET NO. LT01529PCT

Related documentation

Document	Publication Number
QuantStudio™ 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide	MAN0010407
QuantStudio™ Design and Analysis Software v2 User Guide	MAN0018200

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Revision history: Pub. No. MAN0019744

Revision	Date	Description
A.0	01 December 2020	New document.

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APPENDIX 11

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Saliva-Based Molecular Testing for SARS-CoV-2 that Bypasses RNA Extraction

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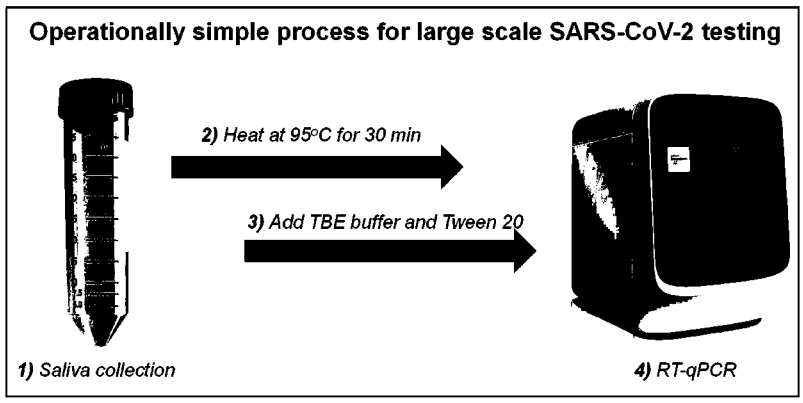
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Abstract

Convenient, repeatable, large-scale molecular testing for SARS-CoV-2 would be a key weapon to help control the COVID-19 pandemic. Unfortunately, standard SARS-CoV-2 testing protocols are invasive and rely on numerous items that can be subject to supply chain bottlenecks, and as such are not suitable for frequent repeat testing. Specifically, personal protective equipment (PPE), nasopharyngeal (NP) swabs, the associated viral transport media (VTM), and kits for RNA isolation and purification have all been in short supply at various times during the COVID-19 pandemic. Moreover, SARS-CoV-2 is spread through droplets and aerosols transmitted through person-to-person contact, and thus saliva may be a relevant medium for diagnosing SARS-CoV-2 infection status. Here we describe a saliva-based testing method that bypasses the need for RNA isolation/purification. In experiments with inactivated SARS-CoV-2 virus spiked into saliva, this method has a limit of detection of 500-1000 viral particles per mL, rivalling the standard NP swab method, and initial studies also show excellent performance with 100 clinical samples. This saliva-based process is operationally simple, utilizes readily available materials, and can be easily implemented by existing testing sites, thus allowing for high-throughput, rapid, and repeat testing of large populations.

Graphical Abstract



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Background

The slow roll-out and inconsistent availability of diagnostic testing for SARS-CoV-2 has hobbled efforts to control the COVID-19 pandemic in many countries. Testing protocols based on the use of nasopharyngeal (NP) swabs as the collection agent, placed in a tube containing viral transport media (VTM), followed by RNA isolation/purification and subsequent analysis by RT-qPCR is currently the most common method (**Figure 1A**).[1,2] While some variant of this process has been implemented worldwide, there are multiple challenges with this workflow. Sample collection using NP swabs requires healthcare workers wearing personal protective equipment (PPE) to collect samples, the swabs can be uncomfortable for the patients during collection, and the swabs and the associated VTM have been in short supply at many times and in most locations. In addition, RNA isolation/purification is another significant bottleneck, both in the time and labor required for this process, and in the availability of the equipment and reagents. All of these components also add to the cost of the testing process.

There is emerging consensus that widespread, frequently repeated testing is necessary for a safer return to activities that are important for society. Given the data suggesting that SARS-CoV-2 can be spread by pre-symptomatic/asymptomatic carriers,[3-6] localized outbreaks could be dramatically reduced or prevented if individuals shedding SARS-CoV-2 could be readily identified and isolated. For example, imagine a testing bubble placed over a group that desires face-to-face interaction – employees of a company, members of a sports team, extended family networks, etc. If all members of the group could be tested for SARS-CoV-2, then isolated, then tested again after an appropriate time increment (likely ~4-5 days, in line with the incubation period for SARS-CoV-2[7,8]), two negative tests would provide confidence for a safer return to activities. Of course, in practice there are challenges with total self-isolation and avoidance of others outside the testing bubble, but the above scenario represents one promising path forward, allowing positive cases to be identified and contained, and reducing the probability that pre-symptomatic/asymptomatic virus shedders unknowingly transmit SARS-CoV-2 to others. Unfortunately, as the size of a group grows larger, widespread and frequent testing for SARS-CoV-2 using the standard testing protocol depicted in **Figure 1A** becomes impractical. For example, it would be untenable to repeatedly test all members of a university in a short time period using this

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process, and thus we were motivated to develop a streamlined, cost-effective, SARS-CoV-2 testing platform that can be realistically scaled to test thousands of individuals a day.

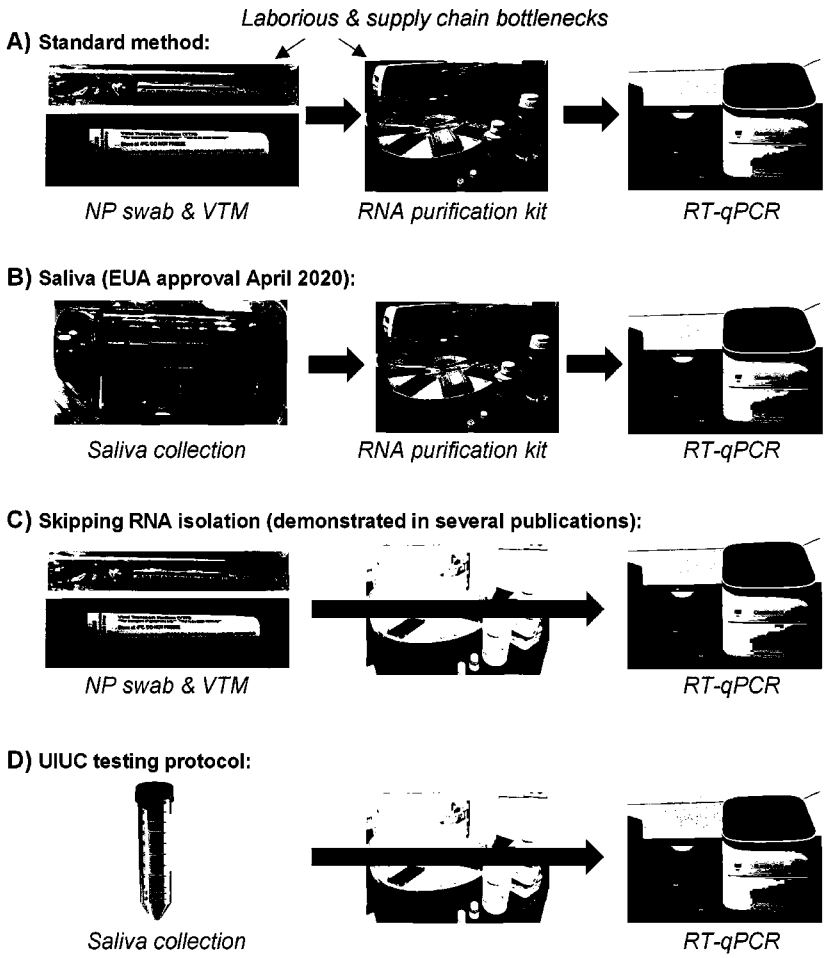


Figure 1. Schematic of SARS-CoV-2 testing. **A)** The current, widely-utilized diagnostic process involves nasopharyngeal (NP) swabs and viral transport media (VTM), followed by RNA extraction and isolation, with RT-qPCR analysis of the samples. NP swabs, VTM, and RNA purification kits have been in short supply at various times. **B)** In April of 2020, saliva was emergency use authorized (EUA) as a diagnostic sample, using RNA extraction and isolation, followed by RT-qPCR. **C)** Other groups have reported direct testing of NP swabs in VTM by RT-qPCR. **D)** The University of Illinois at Urbana-Champaign (UIUC) protocol involves saliva collection in standard 50 mL conical tubes, heating (95°C for 30 min), followed by addition of buffer and analysis by RT-qPCR.

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When considering various sample collection possibilities, saliva is attractive due to the known detection of SARS-CoV-2 through oral shedding, and the potential for rapid and easy self-collection,[9-11] thus minimizing the need for direct healthcare provider-patient contact and consequent conservation of PPE. In addition, a number of recent reports have detailed the detection of SARS-CoV-2 in saliva through the workflow in **Figure 1B**, including a report showing higher viral loads in saliva when compared to matched NP swabs from the same patients.[12] Importantly, saliva (expelled in aerosols and droplets) may be a significant factor in person-to-person transmission of SARS-CoV-2,[10] and it has been suggested that NP swab tests remain positive long after patients are infectious (potentially due to detection of inactive virus or remnants of viral RNA in the NP cavity),[13] whereas SARS-CoV-2 viral loads in saliva are highest during the first week of infection, when a person is most infectious. These data suggest that viral loads in saliva may be a good reflection of the transmission potential of patients infected SARS-CoV-2.[13-15]

While we are unaware of direct SARS-CoV-2 detection from saliva that bypasses RNA isolation/purification, there are several reports of detection from swab/VTM that bypasses RNA isolation/purification (**Figure 1C**).[16-23] With the ultimate goal of providing convenient, scalable, and cost-effective molecular diagnostic testing for >10,000 individuals per day using a single COVID-19 testing center, here we report the discovery of a sensitive saliva-based detection method for SARS-CoV-2 that bypasses RNA isolation/purification (**Figure 1D**). This SARS-CoV-2 testing process and workflow is convenient, simple, rapid, and inexpensive, and can be readily adopted by any testing facility currently using RT-qPCR.

Results

Development of a direct saliva-to-RT-qPCR process for detection of SARS-CoV-2

While SARS-CoV-2 has been identified in the nasopharynx, collecting NP samples is neither trivial nor innocuous, and for repeat testing to track disease progression within a given patient this method may prove unreliable, due to inconsistencies in repeated sampling and potential formation of scar tissue, altogether resulting in possible false-negatives.[24] Compounding these anatomic limitations, the procedure for NP sample collection is invasive, further reducing patient compliance for repeated and serial sampling. Saliva may serve as an important mediator in transmitting SARS-CoV-2 between individuals via droplets and aerosols,[25-27] and

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thus viral loads in saliva may serve as a highly relevant correlate of transmission potential. However, saliva is comprised of constituents that may hinder virus detection by RT-qPCR, such as degradative enzymes. As such, we sought to identify conditions that could take advantage of the many positives of saliva while overcoming potential limit of detection challenges with this collection medium. For the optimization phase of this work we utilized two versions of inactivated SARS-CoV-2, one inactivated through gamma(γ)-irradiation ($5x10^6$ RADs) and one inactivated through heat (65°C, 30 min). For the detection of SARS-CoV-2, we utilized the commercially available TaqPath RT-PCR COVID-19 kit, developed and marketed by Thermo Fisher Scientific. This multiplex RT-qPCR kit targets the ORF1ab (replication), N-gene (nucleocapsid), and S-gene (spike) of SARS-CoV-2. To reduce cost and extend reagent usage, we performed RT-qPCR reactions at half the suggested reaction mix volume.[28]

Heat treatment. Up-front heating of freshly collected saliva samples is attractive as a simple method to inactivate the virus without having to open the collection vessel. Indeed, heat treatment is often used to inactivate saliva patient samples,[29,30] thus conferring added biosafety by decreasing the likelihood of viral transmission via sample handling by personnel. Common conditions for SARS-CoV-2 inactivation are heating at 56-60°C for 30-60 min,[30,31] although other temperature and times have been examined.[30] Using intact, γ-irradiated SARS-CoV-2 spiked into fresh human saliva (that was confirmed to be SARS-CoV-2 negative), we observed dramatic time- and temperature-dependent improvement in SARS-CoV-2 detection by direct RT-qPCR, without the use of RNA extraction. When incubated at ambient temperature (no heat treatment), no SARS-CoV-2 genes were detectable (**Figure 2**). As temperature and incubation time were increased, substantial improvement in virus detection was observed, with 100% identification of all SARS-CoV-2 genes, in all replicate samples, being detected following a 30 min incubation at 95°C. Importantly, a short heating time (5 minutes) at 95°C (as has been examined by others[29,32]) does not allow for sensitive detection; the 30 minute duration is essential, as it is likely that this extended heating inactivates components of saliva that inhibit RT-qPCR. Thus, proper heating of patient samples allows for virus detection without the need for RNA extraction, with the added benefit of inactivating the samples, thus substantially reducing biohazard risks.

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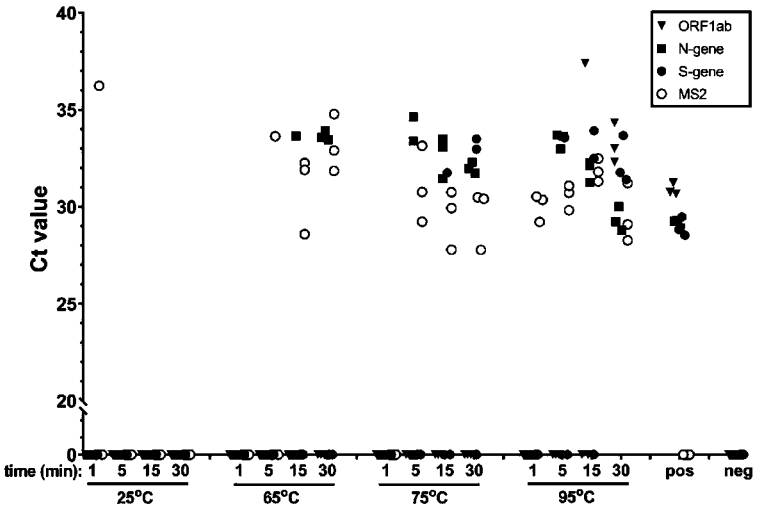


Figure 2. The effect of heat on SARS-CoV-2 detection. γ-irradiated SARS-CoV-2 (from BEI, used at $1.0x10^4$ viral copies/mL) was spiked into fresh human saliva (SARS-CoV-2 negative). Samples diluted 1:1 with 2X Tris-borate-EDTA (TBE) buffer (0.5 mL in 50 mL conical tubes) were incubated at 25°C (ambient temperature), or in a hot water bath at 65°C, 75°C, or 95°C, for 1, 5, 15, or 30 min. All saliva samples were spiked with purified MS2 bacteriophage (1:40 MS2:sample) as an internal control. Virus-spiked saliva samples, a positive control (pos; SARS-CoV-2 positive control, $5.0x10^3$ copies/mL, no MS2) and a negative control (neg; water, no MS2) were directly analyzed by RT-qPCR, in triplicate, for SARS-CoV-2 ORF1ab (green triangle), N-gene (red square), and S-gene (blue circle), and MS2 (open circle). Undetermined Ct values are plotted at 0.

Saliva collection buffer. We next sought to evaluate saliva collection buffers as a means to enhance viral RNA stability, but also to increase uniformity between saliva samples and to decrease sample viscosity. In conjunction with RNA isolation/purification, other groups have utilized protocols whereby saliva was provided by a patient and soon thereafter combined with the collection buffer; reported collection buffers include Phosphate Buffered Saline (PBS),[33] DNA/RNA Shield,[34] and Tris-EDTA (TE).[35] Using intact, γ-irradiated SARS-CoV-2 spiked into fresh human saliva, which was then heat treated at 95°C for 30 min, we observed outstanding virus detection when saliva samples were combined with either Tris-Borate-EDTA (TBE) or TE buffer (**Figure 3A**). Comparable Ct values were observed between TBE and TE buffer, but TE yielded greater variability between individual gene replicates, whereas TBE buffer yielded highly clustered data. In stark contrast, combining saliva with PBS or two commercially available buffers (DNA/RNA Shield, SDNA-1000),

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completely abrogated viral detection, including the MS2 bacteriophage internal control, indicating that these buffers directly interfere with the RT-qPCR reaction itself. TBE, TE, and PBS were further titrated with different concentrations of SARS-CoV-2, where similar trends were observed, namely, greater replicate variability with TE buffer, and no virus detection with PBS (**Supporting Figure 1**). Thus, when saliva samples are combined with TBE buffer to a final working concentration of 1X, SARS-CoV-2 is detectable in saliva without RNA extraction; TE buffer is also suitable but more variability is observed. These findings further suggest that while PBS and commercially available buffers may be appropriate for samples that are processed via RNA extraction, these agents are incompatible with direct saliva-to-RT-qPCR.

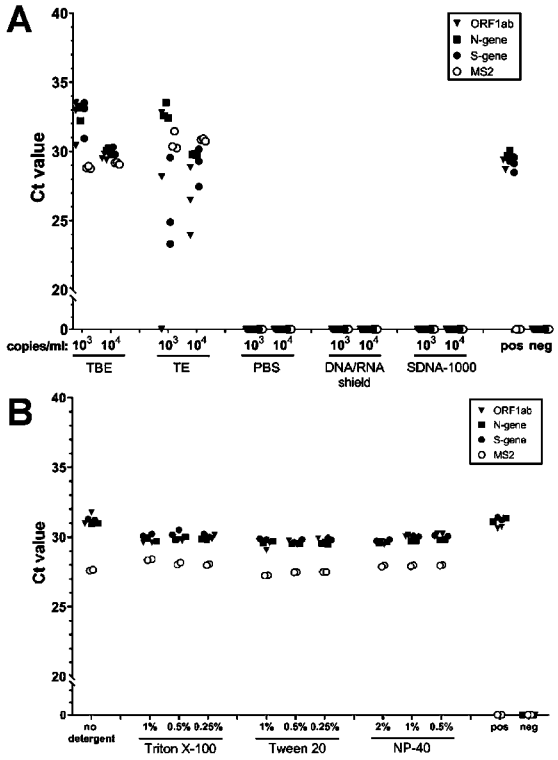


Figure 3. (**A**) The effect of collection buffer on SARS-CoV-2 detection. γ-irradiated SARS-CoV-2 (from BEI, at $1.0x10^3$ or $1.0x10^4$ viral copies/mL) was spiked into fresh human saliva (SARS-CoV-2 negative) and combined at a 1:1 ratio with Tris-Borate-EDTA (TBE), Tris-EDTA (TE), Phosphate Buffered Saline (PBS), DNA/RNA shield (Zymo Research), or SDNA-1000 (Spectrum Solutions) such that the final concentration of each buffer

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was 1X. Samples (0.5 mL in 50 mL conical tubes) were incubated in a hot water bath at 95°C for 30 min. (**B**) Detergent optimization. γ-irradiated SARS-CoV-2 ($1.0x10^4$ viral copies/mL) was spiked into fresh human saliva (SARS-CoV-2 negative) and combined 1:1 with TBE buffer at a final working concentration of 1X. Samples were treated with detergents (Triton X-100, 1%, 0.5%, 0.25%; Tween 20, 1%, 0.5%, 0.25%; NP-40, 2%, 1%, 0.5%) after heating at 95°C for 30 min. All saliva samples were spiked with purified MS2 bacteriophage (1:40 MS2:sample) as an internal control. Virus-spiked saliva samples, a positive control (pos; SARS-CoV-2 positive control, $5.0x10^3$ copies/mL, no MS2) and a negative control (neg; water, no MS2) were directly analyzed by RT-qPCR, in triplicate, for SARS-CoV-2 ORF1ab (green triangle), N-gene (red square), and S-gene (blue circle), and MS2 (open circle). Undetermined Ct values are plotted at 0.

Sample additives. In addition to saliva collection buffers, various additives have been explored for their ability to enhance SARS-CoV-2 detection.[1,36-38] Therefore, detergents, including Triton X-100, Tween 20, and NP-40 (**Figure 3B**), as well as various RNA stabilizing agents, including RNase inhibitor, carrier RNA, glycogen, TCEP, proteinase K, bovine serum albumin (BSA), RNAlater, and PBS-DTT (**Supporting Figure 2**) were examined. Notably, modest improvements in viral detection were observed with all detergents tested (~2 Ct, **Figure 3B**) and with addition of carrier RNA, RNase inhibitor, and BSA (**Supporting Figure 2**), These additives slightly improve virus detection, without interfering with RT-qPCR; in addition, if clinical saliva specimens are especially viscous, addition of detergent may improve ease of sample handling. However, inclusion of detergents prior to heat treatment inhibited viral detection, emphasizing the importance of adding detergents after heat treatment, if they are to be included (**Supporting Figure 3**). Of the detergents tested, Tween 20 was chosen for incorporation into the standard sample processing protocol, given its ease of handling and cost. When samples were treated with Tween 20 and TBE (alone or in combination, either before or after heating) the ideal workflow for virus detection, as defined by the lowest Ct values with the greatest clustering of individual replicates, was TBE buffer before heating, and Tween 20 after heating (**Supporting Figure 3**). However, it is important to note that comparable results were obtained when TBE was added after heating (**Supporting Figure 3**), suggesting flexibility in when TBE buffer can be included during sample processing. Altogether, the safest and most streamlined protocol would be: collection of saliva samples, heat at 95°C for 30 min, add TBE buffer and Tween 20, followed by RT-qPCR.

Limit of detection. Using the optimized protocol of addition of TBE (or TE) buffer at a 1:1 ratio with saliva, followed by heat treatment at 95°C for 30 min and addition of Tween 20 to a final concentration of 0.5%, the limit of detection (LOD) was determined. Other reports have suggested that SARS-CoV-2 is shed into

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saliva at a remarkably wide range from 10,000-10,000,000,000 copies/mL.[12,26] While the LOD of SARS-CoV-2 approved diagnostic methods can vary considerably (500-80,000 viral copies/mL[39]) and are not always reported, the best LOD values for SARS-CoV-2 using RNA extraction protocols appear to be approximately 1000 copies/mL.[28] Similarly, a LOD of 5610 copies/mL was found for SARS-CoV-2 detection in saliva using RNA purification.[12] To determine the LOD for this new direct protocol (saliva→RT-qPCR), a side-by-side comparison was conducted of intact, γ-irradiated SARS-CoV-2 spiked into fresh human saliva compared to a process that includes RNA isolation/purification. As shown in **Figure 4**, comparable LOD measurements were observed, with LOD of ~500 viral copies/mL for both the direct process with addition of Tween 20 and TBE buffer, and the process using RNA purification. Similar results were observed with heat-inactivated SARS-CoV-2, whereby the LOD was measured to be 5000 viral copies/mL for both RNA extraction of saliva samples and direct saliva-to-RT-qPCR, with greater detection if the virus was directly analyzed in water (**Supporting Figure 4**).

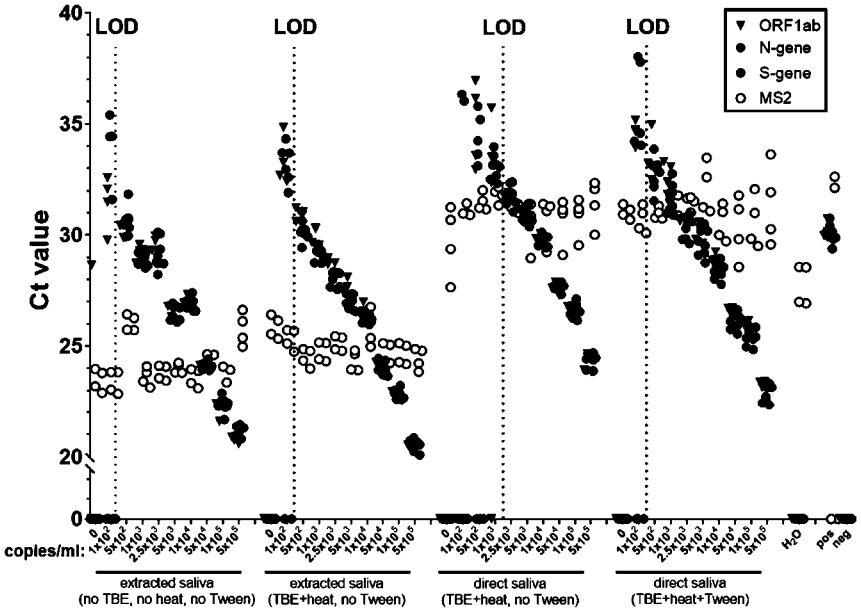


Figure 4. Limit of Detection (LOD) for assessment of SARS-CoV-2 from saliva, comparing a process utilizing RNA isolation/purification to one that bypasses RNA isolation/purification. γ-irradiated SARS-CoV-2 was

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spiked into fresh human saliva (SARS-CoV-2 negative), with or without TBE buffer(1X) at $1.0x10^2$, $5.0x10^2$, $1.0x10^3$, $2.5x10^3$, $5.0x10^3$, $1.0x10^4$, $5.0x10^4$, $1.0x10^5$, and $5.0x10^5$ viral copies/mL. Samples were incubated at 95°C for 30 min, then combined with or without Tween 20 (0.5%). All saliva samples were spiked with purified MS2 bacteriophage (1:40 MS2:sample) as an internal control. Virus-spiked saliva samples were either processed for RNA extraction followed by RT-qPCR (purified RNA), or directly analyzed by RT-qPCR (direct saliva). All samples, including a positive control (pos; SARS-CoV-2 positive control, $5.0x10^3$ copies/mL, no MS2) and a negative control (neg; water, no MS2) were analyzed by RT-qPCR, in triplicate, for SARS-CoV-2 ORF1ab (green triangle), N-gene (red square), and S-gene (blue circle), and MS2 (open circle). Undetermined Ct values are plotted at 0. The limit of detection (LOD) is indicated by the dotted vertical line.

As the TaqPath/MasterMix RT-qPCR reagents from ThermoFisher provide the necessary specificity for SARS-CoV-2 detection in a simplified workflow, this system was utilized for all the experiments described above. However, we have also assessed the Centers for Disease Control and Prevention (CDC)-approved primers and probes for SARS-CoV-2 N1 and N2 genes, and the human RNase P (RP) gene control in this direct saliva-to-RT-qPCR protocol, and the results show that these primers give comparable LOD values, with 5000 viral copies/mL using heat-inactivated SARS-CoV-2, and 500 viral copies/mL using γ-irradiated SARS-CoV-2 (**Supporting Figure 5**). These findings further illustrate that our optimized protocol may be used with comparable detection across multiple analytical platforms. Altogether, these findings indicate that the optimized protocol (heat treatment of saliva samples at 95°C for 30 min / addition of TBE buffer and Tween 20) yields a LOD that is comparable to reported clinical viral shedding concentrations in oral fluid, thus emphasizing the translatability of the protocol to detecting SARS-CoV-2 in patient samples.

Sample handling optimization. In preparation for clinical samples and real-world testing, we first evaluated the ability to detect spiked inactivated virus in samples that were stored at varying temperatures (ambient (25°C), 4°C, -20°C, and -80°C), for varying lengths of time (≤24 hrs). Most importantly, at room temperature and at 4°C samples processed after 1 hr showed little difference from those processed after 24 hr storage, suggesting considerable flexibility in processing time (**Supporting Figure 6**). Some increased variability between individual gene replicates and loss of signal was observed with prolonged storage and freeze/thaw cycles (**Supporting Figure 6**).

Next, evaluation was made of the effect of sample volume in the saliva collection vessels (50 mL conical tubes) on viral detection, after heating at 95°C for 30 min in a hot water bath, due to concerns of evaporation of smaller samples and incomplete heating of larger samples. No appreciable difference was observed across

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the anticipated range of clinical saliva sample volumes (0.5-5 mL), indicating that sample volume does not impact virus detection (**Supporting Figure 7**). Furthermore, if samples are transferred to smaller vessels for more efficient long-term cold storage (1.5 mL microcentrifuge tubes), no appreciable differences in virus detection between different volumes is anticipated (**Supporting Figure 7**). Finally, as clinical saliva samples can sometimes contain particulates, we next evaluated whether removal of the particulates via centrifugation affected viral detection (**Supporting Figure 8**). Notably, if samples were centrifuged, with the resultant supernatant being used for direct RT-qPCR, the LOD was approximately 10-fold worse, with fewer individual gene replicates being detected at lower viral copy numbers (**Supporting Figure 8**). Therefore, we recommend avoiding centrifugation of samples if possible. Altogether, these findings suggest that (1) saliva samples are stable under varying storage conditions, (2) the volume of sample heated with collection vessels does not affect viral detection, and (3) centrifugation of samples should be avoided for direct saliva-to-RT-qPCR testing of SARS-CoV-2.

LOD reproducibility. In order to evaluate the robustness of the optimized direct saliva-to-RT-qPCR approach, the LOD of 1000 SARS-CoV-2 viral copies/mL was measured in 30 independent replicate samples (**Figure 5**). γ-irradiated SARS-CoV-2 was spiked into fresh saliva from two healthy donors, and two commercially available saliva sources. Across all replicates, these samples with 1000 viral copies/mL were consistently detected (all three viral genes), further testifying to the ability of direct saliva-to-RT-qPCR to detect SARS-CoV-2. In order to validate the specificity of our detection system to SARS-CoV-2, saliva was spiked with or without SARS-CoV-2 (γ-irradiated virus, synthetic N-transcript), two other human coronaviruses (OC43, 229E), SARS and MERS synthetic RNA, and human RNA (extracted from HEK 293 cells). Among these samples, SARS-CoV-2 genes were only detected in the positive control, and SARS-CoV-2 samples, further supporting the specificity of the detection platform for SARS-CoV-2 (**Supporting Figure 9**).

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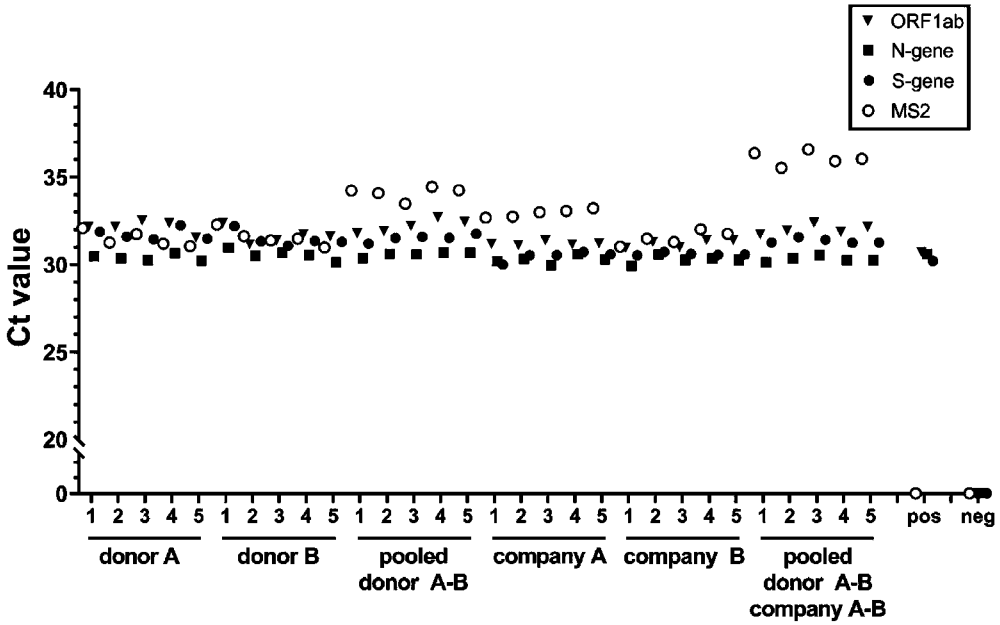


Figure 5. Limit of Detection (LOD) reproducibility. γ-irradiated SARS-CoV-2 was spiked into human saliva (SARS-CoV-2 negative), sourced fresh from two healthy donors, and purchased from two companies, in 1X TBE buffer at $1.0x10^3$ viral copies/mL. Samples were incubated at 95°C for 30 min, then Tween 20 was added to a final concentration of 0.5%. All saliva samples were spiked with purified MS2 bacteriophage (1:40 MS2:sample) as an internal control. Virus-spiked saliva samples were directly analyzed by RT-qPCR (direct saliva). All samples, including a positive control (pos; SARS-CoV-2 positive control, $5.0x10^3$ copies/mL, no MS2) and a negative control (neg; water, no MS2) were analyzed by RT-qPCR, in replicates of 5, for SARS-CoV-2 ORF1ab (green triangle), N-gene (red square), and S-gene (blue circle), and MS2 (open circle). Undetermined Ct values are plotted at 0.

Clinical validation of direct saliva-to-RT-qPCR for diagnosis of SARS-CoV-2

Our findings support an optimized SARS-CoV-2 diagnostic approach that increases accessibility to testing by using saliva (rather than NP swabs) and eliminates the need for RNA extraction (thus saving time and resources). We next sought to assess our protocol with clinical samples. Although the changes in viral load in the NP cavity and in saliva over time are unknown, there is reason to believe they are different,[40,41] so exact concordance between the two samples might not be expected; detection in saliva can provide complementary information to that in the NP cavity.

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To evaluate the ability of the direct saliva-to-RT-qPCR approach to detect SARS-CoV-2 in clinical patient specimens, saliva was collected contemporaneously with NP swabs from 100 individuals using the following protocol: After saliva collection, TE was added at a 1:1 ratio, and samples were frozen for over a week before processing. For the evaluation, samples were thawed, 10X TBE buffer was added to a final concentration of 1X, heated at 95°C for 30 min, cooled to room temperature, and Tween 20 was added to a final concentration of 0.5%, followed by direct RT-qPCR. Given biological complexity in clinical samples, variabilities in signal detection based on viral load and gene target length (ORF1ab > S > N) may occur; therefore, a given result was interpreted as positive if one or more gene targets were detected, and negative if no gene targets were detected. Furthermore, a result was considered valid if all gene targets were detected in the SARS-CoV-2 positive control and no gene targets were detected in the negative control. A notable power in the context of a multiplex system is the ability to evaluate three independent viral genes in a single reaction, rather than relying upon multiple probes across different reactions for a single viral gene (as is used in other systems). One of the benefits of saliva-based testing is the possibility of frequent and easy retesting of samples and of individuals, and as such duplicate testing (testing of the same saliva sample two different times) was utilized for this study.

Of the 100 samples analyzed, 9 were positive for SARS-CoV-2 as assessed by NP swab, and upon duplicate testing the direct saliva-to-RT-qPCR process identified the same 9 samples as positive, with 8 of 9 saliva samples positive in both of the replicates. All 91 samples identified as negative by NP swab were also negative via saliva testing, although in one of these samples one of the duplicate runs was positive, but was negative upon re-tests (**Figure 6, Table 1**). Even though these samples were not run under the fully optimized protocol, this initial testing of clinical samples using direct saliva-to-RT-qPCR showed excellent performance. When testing samples a single time, it was 88.9% sensitive and 98.9% specific for SARS-CoV-2, with an 11.1% false negative and 1.1% false positive rate, and 88.9% positive and 98.9% negative predictive value. Using duplicate testing of samples, sensitivity and specificity, and positive and negative predictive values, all increased to 100%, and the false negative and positive rates decreased to 0%.

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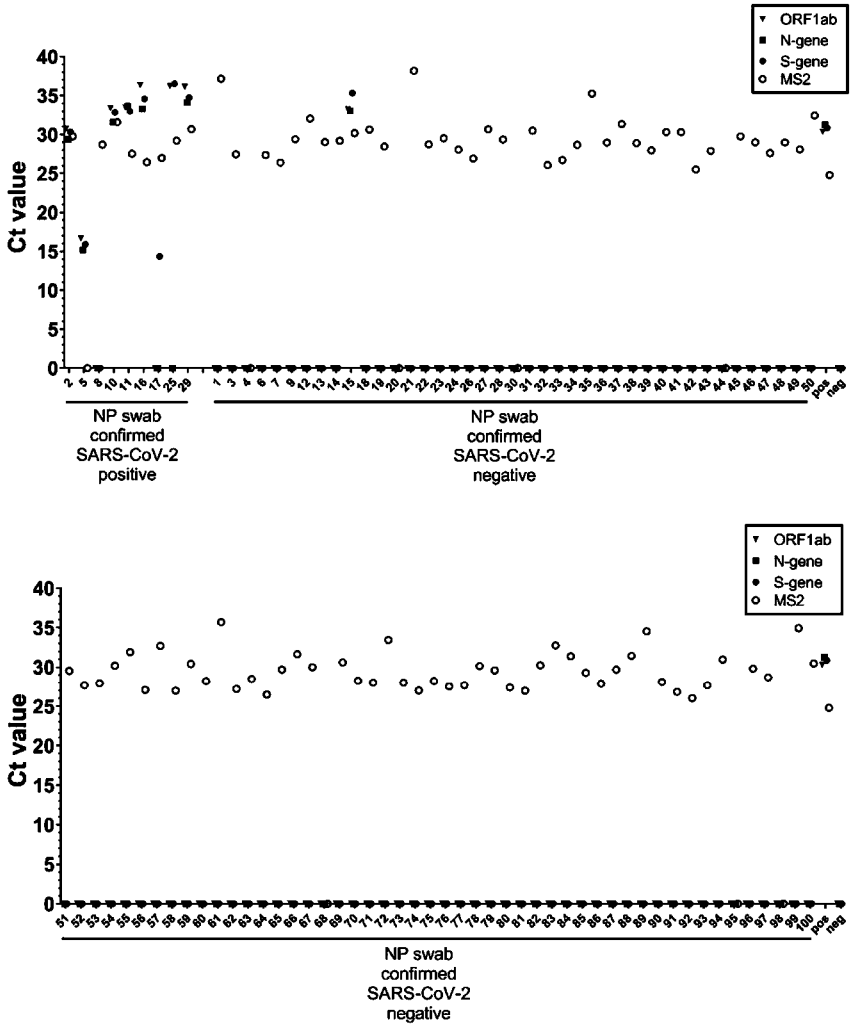


Figure 6. Assessment of clinical samples. Saliva samples from 9 SARS-CoV-2 positive and 91 SARS-CoV-2 negative patients (as judged by NP swabs in VTM with RNA extraction) had TE buffer added to them (at a 1:1 ratio) and were frozen for over a week. Upon thawing, 10X TBE buffer was added to the samples at a final concentration of 1X, heated at 95°C for 30 min, cooled to room temp, and Tween 20 was added to a final concentration of 0.5%. All saliva samples were spiked with purified MS2 bacteriophage (1:40 MS2:sample) as an internal control. Saliva samples were directly analyzed by RT-qPCR. All samples, including a positive control (pos; SARS-CoV-2 positive control, $5.0x10^3$ copies/mL) and a negative control (neg; water) were analyzed by RT-qPCR, in singlet, for SARS-CoV-2 ORF1ab (green triangle), N-gene (red square), and S-gene (blue circle), and MS2 (open circle). This figure shows one of the two replicates. Undetermined Ct values are plotted at 0.

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Table 1. Clinical evaluation of direct saliva-to-RT-qPCR

NP-swab confirmed SARS-CoV-2 positive			
ID	NP swab result	direct saliva-to-RT-qPCR result	re-test
2	POS	POS	POS
5	POS	POS	POS
8	POS	NEG	POS
10	POS	POS	POS
11	POS	POS	POS
16	POS	POS	POS
17	POS	POS	POS
25	POS	POS	POS
29	POS	POS	POS

NP-swab confirmed SARS-CoV-2 negative			
ID	NP swab result	direct saliva-to-RT-qPCR result	re-test
1	NEG	NEG	NEG
3	NEG	NEG	NEG
4	NEG	NEG	NEG
6	NEG	NEG	NEG
7	NEG	NEG	NEG
9	NEG	NEG	NEG
12	NEG	NEG	NEG
13	NEG	NEG	NEG
14	NEG	NEG	NEG
15	NEG	POS	NEG
18	NEG	NEG	NEG
19	NEG	NEG	NEG
20	NEG	NEG	NEG
21	NEG	NEG	NEG
22	NEG	NEG	NEG
23	NEG	NEG	NEG
24	NEG	NEG	NEG
26	NEG	NEG	NEG
27	NEG	NEG	NEG
28	NEG	NEG	NEG
30	NEG	NEG	NEG
31	NEG	NEG	NEG
32	NEG	NEG	NEG
33	NEG	NEG	NEG
34	NEG	NEG	NEG
35	NEG	NEG	NEG
36	NEG	NEG	NEG
37	NEG	NEG	NEG
38	NEG	NEG	NEG
39	NEG	NEG	NEG
40	NEG	NEG	NEG
41	NEG	NEG	NEG
42	NEG	NEG	NEG
43	NEG	NEG	NEG
44	NEG	NEG	NEG
45	NEG	NEG	NEG
46	NEG	NEG	NEG
47	NEG	NEG	NEG
48	NEG	NEG	NEG
49	NEG	NEG	NEG
50	NEG	NEG	NEG

NP-swab confirmed SARS-CoV-2 negative			
ID	NP swab result	direct saliva-to-RT-qPCR result	re-test
51	NEG	NEG	NEG
52	NEG	NEG	NEG
53	NEG	NEG	NEG
54	NEG	NEG	NEG
55	NEG	NEG	NEG
56	NEG	NEG	NEG
57	NEG	NEG	NEG
58	NEG	NEG	NEG
59	NEG	NEG	NEG
60	NEG	NEG	NEG
61	NEG	NEG	NEG
62	NEG	NEG	NEG
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92	NEG	NEG	NEG
93	NEG	NEG	NEG
94	NEG	NEG	NEG
95	NEG	NEG	NEG
96	NEG	NEG	NEG
97	NEG	NEG	NEG
98	NEG	NEG	NEG
99	NEG	NEG	NEG
100	NEG	NEG	NEG

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Discussion

Comparison of NP swab and saliva-based testing. When seeking to develop a SARS-CoV-2 molecular diagnostic protocol suitable for testing >10,000 individuals a day, the ease with which saliva can be collected, and the known presence of the virus in saliva makes it highly desirable as the sample medium. As a diagnostic tool, such testing has the additional advantage of making assessments directly from an oral fluid that may be a culprit in transmission of SARS-CoV-2.[10] Unfortunately, only a handful of studies have examined the viral load dynamics over time for saliva and NP swab samples.[40,41] While these studies support the notion that SARS-CoV-2 tends to be at its highest level in saliva during the first week of infection, more information is needed on this important topic. In contrast, studies have shown that while live virus can no longer be cultured from patients 10 days after symptom onset,[42] NP swabs continue to be positive after a patient is in the convalescent phase and no longer infectious.[13] As such, it is quite possible that differences observed in studies comparing SARS-CoV-2 levels in saliva and NP swabs are real, and not an artifact of different testing sensitivities; while in general concordance between the NP swab and saliva testing has been high in other studies (87%,[40] 92%,[14] 100%[43]), results will likely depend on what point during infection a patient is sampled.

Direct saliva-to-RT-qPCR process, key advances and remaining limitations. The direct saliva-to-RT-qPCR method described herein, bypassing NP swabs, VTM, and RNA isolation/purification, was enabled by a handful of key discoveries. First, the time and duration of heating the saliva sample is critical. Standard protocols for heat inactivation of SARS-CoV-2 call for heating at ~60°C for 30 minutes;[30,31] while these conditions inactivate the virus, they do not allow for successful SARS-CoV-2 detection via direct RT-qPCR, likely because of the persistence of as-yet-unidentified factors in saliva that are inhibitory to RT-qPCR. Heating at 95°C for 30 minutes likely inactivates these inhibitory components and allows for excellent SARS-CoV-2 detection in this direct process that bypasses RNA isolation/purification. Second, while TE buffer performs well, consistent with another report successfully using TE to extract dry NP swabs,[17] TBE buffer provides more reliability and consistency in our direct saliva-to-RT-qPCR detection of SARS-CoV-2. Finally, the addition of the non-ionic detergent Tween-20 also helped improve detection of SARS-CoV-2, possibly by facilitating the opening of the viral capsid to allow the release of RNA to provide sufficient template for RT-qPCR detection.

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Our preliminary assessment of clinical samples is very promising, especially given that these samples were not collected and processed under the optimized protocol (they were collected before our discovery of the benefits of TBE buffer and Tween 20); with these samples TE buffer was added to the sample, and they were frozen for over a week before processing. However, even under this non-optimized workflow we were able to identify all 9 NP swab positives with duplicate runs of the samples. Next steps are to perform similar head-to-head comparisons between the NP swab-based method and our optimized workflow with additional clinical samples.

Supply chain, costs, and next-generation technology. A major benefit of the simple workflow (see Graphical Abstract) detailed herein is its ability to be adopted by any diagnostic laboratory currently using RT-qPCR in SARS-CoV-2 testing. In addition to the time savings and major logistical benefits of using saliva and bypassing RNA isolation/purification, our analysis of the costs of all reagents/disposables for this process amounts to ~$10 per test, the bulk of which are the TaqPath/MasterMix. This cost could drop further if samples are pooled before RT-qPCR. Pooling considerations will necessarily be informed by data on the expected positive rate in the population to be tested, and also the relationship between viral load and infectivity;[44-46] while one recent study showed that live SARS-CoV-2 could not be cultured from samples containing less than 1,000,000 viral copies per mL,[42] more information is needed. And, while there is no indication that TaqPath/MasterMix will be limited by the supply chain, we show that this process and workflow is also compatible with other primer sets, such as the N1 and N2 primers and probes from the CDC. In the future, development of analogous saliva-based processes that bypass RNA isolation/purification can be envisioned for alternative back-end detection technologies, such as the LAMP method,[2,47] which if successful would result in an even shorter overall time from sample collection to results.

In summary, described herein is a sensitive diagnostic method for SARS-CoV-2 that is operationally simple, bypasses supply chain bottlenecks, evaluates a clinically relevant infectious fluid, is appropriate for large scale repeat testing, is cost effective, and can be readily adopted by other laboratories. Large scale SARS-CoV-2 testing will be a powerful weapon in preventing spread of this virus and helping to control the COVID-19 pandemic.

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Acknowledgement

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Materials and Methods

Acquisition and processing of clinical samples

All clinical samples from study participants were collected in accordance with University of Illinois at Urbana-Champaign (UIUC) IBC-approved protocol number 4604 and IRB-approved protocol number 20CRU3150. Saliva in 1:1 1X TE buffer and discarded VTM samples collected from 100 adults at the Carle Foundation Hospital Drive-thru COVID-19 testing center were collected and frozen at -80°C for over a week. Upon thawing, 10X TBE buffer was added to the samples to a final concentration of 1X, heated at 95°C for 30 min, cooled to room temperature, and Tween 20 was added to a final concentration of 0.5%. The optimized direct saliva-to-RT-qPCR approach was compared to detection of SARS-CoV-2 from nasopharyngeal (NP) swab in VTM performed at the Carle Foundation Hospital. In all studies conducted, researchers were blinded to the results obtained from clinical RT-qPCR tests performed on NP swabs at the Carle Foundation Hospital.

Collection and processing of fresh saliva from healthy donors

Fresh saliva was collected from healthy individuals in 50 mL conical tubes (BD Falcon) in accordance with University of Illinois at Urbana-Champaign IBC-approved protocol numbers 4604 and 4589. In some experiments, pooled saliva from healthy donors was purchased from Lee BioSolutions, Inc. (CN 991-05-P) and Innovative Research (CN IRHUSL50ML). Saliva was diluted at a 1:1 ratio with either TBE buffer (100mM Tris-HCl pH8.0, 90mM boric acid, and 1mM EDTA) or TE buffer (10mM Tris-HCl pH8.0 and 1mM EDTA) buffer. In some experiments, Phosphate Buffered Saline (PBS), DNA/RNA Shield (Zymo Research), and SDNA-1000

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(Spectrum Solutions), were also tested at final working concentrations of 2X, 1.5X, 1X, and 0.5X. Known amounts of the SARS-CoV-2 inactivated virus (BEI) were spiked into saliva samples. Samples were incubated in a hot water bath at 95°C for 30 min. All saliva samples were spiked with purified MS2 bacteriophage (1:40 MS2:sample) as an internal control. In some experiments, RNA extraction was performed on 200 µL saliva (+/- virus) using MagMax Viral/Pathogen II Nucleic Acid Isolation Kit (Applied Biosciences CN A48383) following the manufacturer's protocol. Extracted RNA was eluted from magnetic beads in 50µl UltraPure DNase/RNase-free distilled water (Ambion CN 10977023). RNA concentration of eluted RNA was measured using Qubit RNA Broad Range (BR) assay kit (Fisher Scientific).

SARS-CoV-2 inactivated virus and human coronaviruses

In most experiments, fresh pooled saliva diluted 1:1 in TBE buffer (1x final concentration) were spiked with either gamma-irradiated (BEI cat# NR-52287, Lot no. 70033322) or heat-inactivated (BEI cat# NR-52286, Lot no. 70034991) SARS-CoV-2 virions. The reported genome copy number pre-inactivation for γ-irradiated and heat-inactivated SARS-CoV-2 are $1.7x10^9$ and $3.75x10^8$ genome equivalents/mL, respectively, for the specified lot numbers. The following reagent was deposited by the Centers for Disease Control and Prevention and obtained through BEI Resources, NIAID, NIH: SARS-Related Coronavirus 2, Isolate USA-WA1/2020, Gamma-irradiated, NR-52287, and heat-inactivated, NR-52286. Seasonal human coronaviruses (OC43 and 229E strains) were obtained from the World Reference Center for Emerging Viruses and Arboviruses at UTMB.

Genomic RNA for SARS-Related Coronavirus 2 (Isolate USA-WA1/2020), NR-52285, was obtained from BEI Resources. In addition, the 2019-nCoV_N_Positive Control (CN 10006625), SARS-CoV Control (CN 10006624), and MERS-CoV Control (CN 10006623) synthetic RNA transcripts were purchased from Integrated DNA Technologies.

All virus stocks and RNA transcripts were aliquoted in small volumes and stored at -70°C. Stocks were serially diluted to the correct concentration in RNase-free water on the day of experimentation.

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RT-qPCR assay

We performed a multiplex RT-qPCR assay using the TaqPath RT-PCR COVID-19 kit (Thermo Fisher CN A47814) together with the TaqPath 1-step master mix – No ROX (Thermo Fisher CN A28523). To reduce cost, RT-qPCR reactions were prepared at half the suggested reaction mix volume (7.5 µL instead of 15 µL). 10 µL of either saliva in TBE buffer or extracted RNA were used as templates for the RT-qPCR reaction. All saliva samples used for pre-clinical studies were spiked with purified MS2 bacteriophage (1:40 MS2:sample) as an internal control prior to analysis by RT-qPCR. For clinical samples, MS2 was added to the preparation of the reaction mix (1µL MS2 per reaction). COVID-19 positive control RNA at 25 genomic copies/µL was used. Negative control is UltraPure DNase/RNase-free distilled water (Ambion CN 10977023). All RT-qPCR reactions were performed in 0.2 mL 96-well reaction plates in a QuantStudio 3 system (Applied Biosciences). The limit of detection (LOD) of the assay was performed by serial dilution of γ-irradiated SARS-CoV-2 (0-$5.0x10^5$ viral copies/mL) used to spike pooled fresh saliva samples. The RT-qPCR was run using the standard mode, consisting of a hold stage at 25°C for 2 min, 53°C for 10 min, and 95°C for 2 min, followed by 40 cycles of a PCR stage at 95°C for 3 sec then 60°C for 30 sec; with a 1.6°C/sec ramp up and ramp down rate.

In some experiments, the CDC-approved assay was used to validate our data using the TaqPath 1-step mix (Thermo Fisher CN A15300). Primers and probes targeting the N1, N2, and RP genes were purchased from Integrated DNA Technologies as listed: nCOV_N1 Forward Primer Aliquot (CN 10006830), nCOV_N1 Reverse Primer Aliquot (CN 10006831), nCOV_N1 Probe Aliquot (CN 10006832), nCOV_N2 Forward Primer Aliquot (CN 10006833), nCOV_N2 Reverse Primer Aliquot (CN 10006834), nCOV_N2 Probe Aliquot (CN 10006835), RNase P Forward Primer Aliquot (CN 10006836), RNase P Reverse Primer Aliquot (CN 10006837), RNase P Probe Aliquot (CN 10006838). The 2019-nCoV_N_Positive Control (IDT CN 10006625) was used as positive control at 50 copies/µL dilution.

Detergent optimization

γ-irradiated SARS-CoV-2 ($1.0x10^4$ viral copies/mL) was spiked into fresh human saliva (SARS-CoV-2 negative) and combined 1:1 with Tris-Borate-EDTA buffer (TBE) at a final working concentration of 1X.

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Samples were treated with varying concentrations of detergents (Triton X-100 (Fisher Scientific), Tween 20 (Fisher Scientific), NP-40 (Fisher Scientific)) before or after heating at 95°C for 30 min. All saliva samples were spiked with purified MS2 bacteriophage (1:40 MS2:sample) as an internal control prior to analysis by RT-qPCR (Fisher TaqPath COVID-19 Combo kit, QuantStudio 3).

Sample volume heat treatment optimization

γ-irradiated SARS-CoV-2 ($1.0x10^4$ viral copies/mL; BEI) was spiked into fresh human saliva (SARS-CoV-2 negative) and combined 1:1 with Tris-Borate-EDTA buffer (TBE) at a final working concentration of 1X. The sample was distributed into either 50 mL conical (BD Falcon) or 1.5 mL microfuge tubes (Ambion), at either 10% (5 mL in 50 mL conical, 150 µL in 1.5 ml microfuge), 5% (2.5 ml in 50 mL conical, 75 µL in 1.5 mL microfuge), or 1% (0.5 mL in 50 mL conical, 15 µL in 1.5 mL microfuge) the vessel storage capacity. Samples were incubated in a hot water bath at 95°C for 30 min. All saliva samples were spiked with purified MS2 bacteriophage (1:40 MS2:sample) as an internal control prior to analysis by RT-qPCR (Fisher TaqPath COVID-19 Combo kit, QuantStudio 3).

Sample buffer additive optimization

γ-irradiated SARS-CoV-2 ($1.0x10^4$ viral copies/mL) was spiked into fresh human saliva (SARS-CoV-2 negative) and combined 1:1 with Tris-Borate-EDTA buffer (TBE) at a final working concentration of 1X in 50 mL conical tubes (BD Falcon). Samples (1.0 mL in 50 mL conical tubes) were incubated in a hot water bath at 95°C for 30 min. Following heat treatment, virus-spiked saliva was aliquoted in 1.5 mL tubes and combined with various RNA stabilizing agents to a final volume of 40 µL. Additives include RNaseI (1 U/µL), carrier RNA (0.05 µg/mL), glycogen (1 µg/µL), TCEP/EDTA (1X), Proteinase K (5 µg/µL), RNase-free BSA (1.25 mg/ml), RNAlater (1:1 ratio in place of TBE), or PBS-DTT (6.5 mM DTT in PBS, diluted 1:1 in place of TBE). All saliva samples were spiked with purified MS2 bacteriophage (1:40 MS2:sample) as an internal control prior to analysis by RT-qPCR (Fisher TaqPath COVID-19 Combo kit, QuantStudio 3).

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Saliva stability optimization

Pre-aliquoted γ-irradiated SARS-CoV-2 ($1.0x10^4$ viral copies/mL) was spiked into pre-aliquoted fresh human saliva (SARS-CoV-2 negative) and combined with Tris-Borate-EDTA buffer (TBE), at a final working concentration of 1X. Samples (0.5 mL in 50 mL conical tubes) were stored at 25°C (ambient temperature), 4°C, -20°C, or -80°C for 1, 2, 4, 8, 12, and 24 hours. All saliva samples were spiked with purified MS2 bacteriophage (1:40 MS2:sample) as an internal control prior to analysis by RT-qPCR (Fisher TaqPath COVID-19 Combo kit, QuantStudio 3).

Data analysis

Following completion of RT-qPCR, data were processed using QuantStudio Design and Analysis Software (version 1.2). Cycle threshold (Ct) values were plotted as single replicate values on a scatter plot, using GraphPad Prism 8 (version 8.4.2). Sensitivity, specificity, false positive, false negative, positive predictive values, and negative predictive values were calculated using the current standard for SARS-CoV-2 detection (NP swabs in VTM with RNA extraction) as confirmation of true disease positive and disease negative status.

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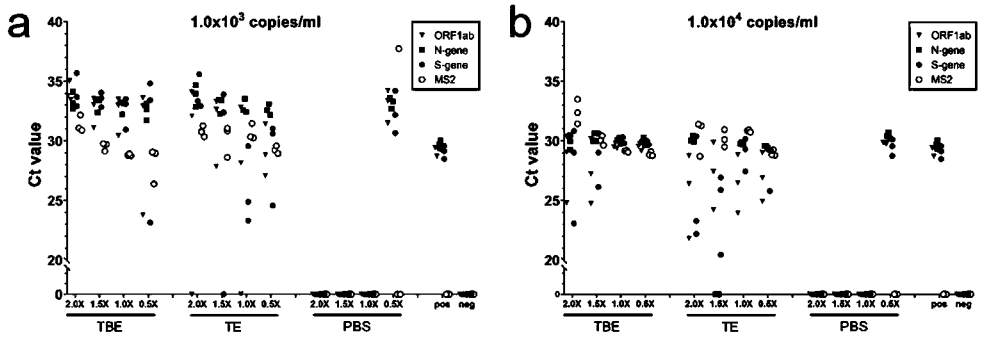
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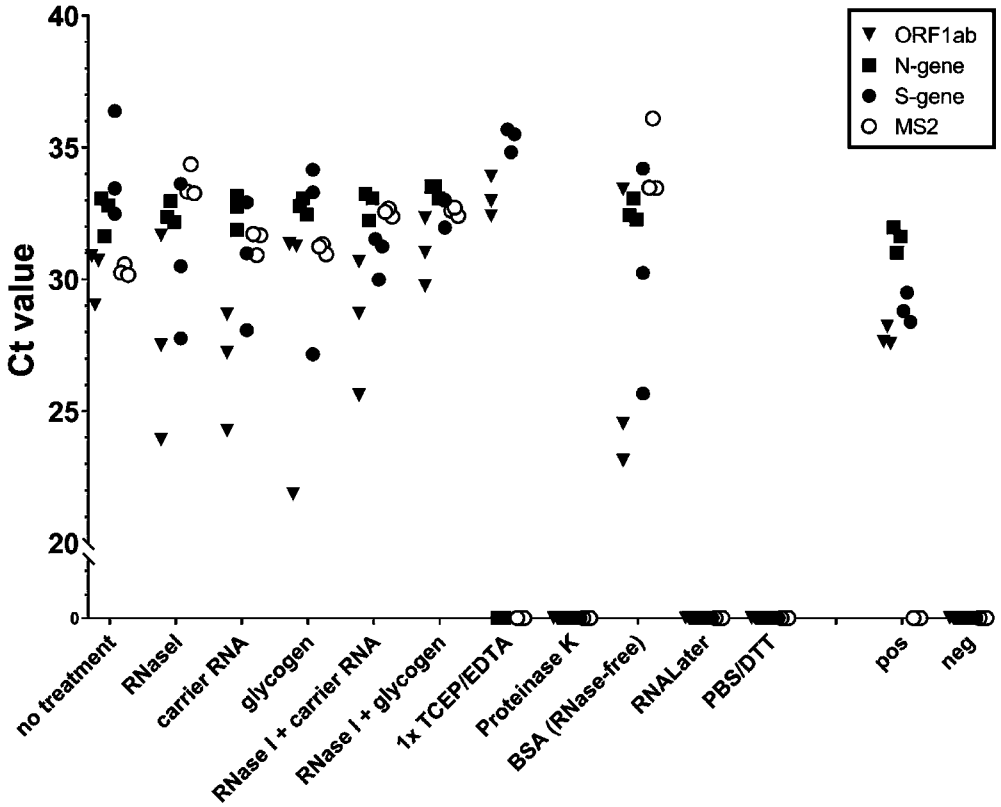
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Supporting Figures



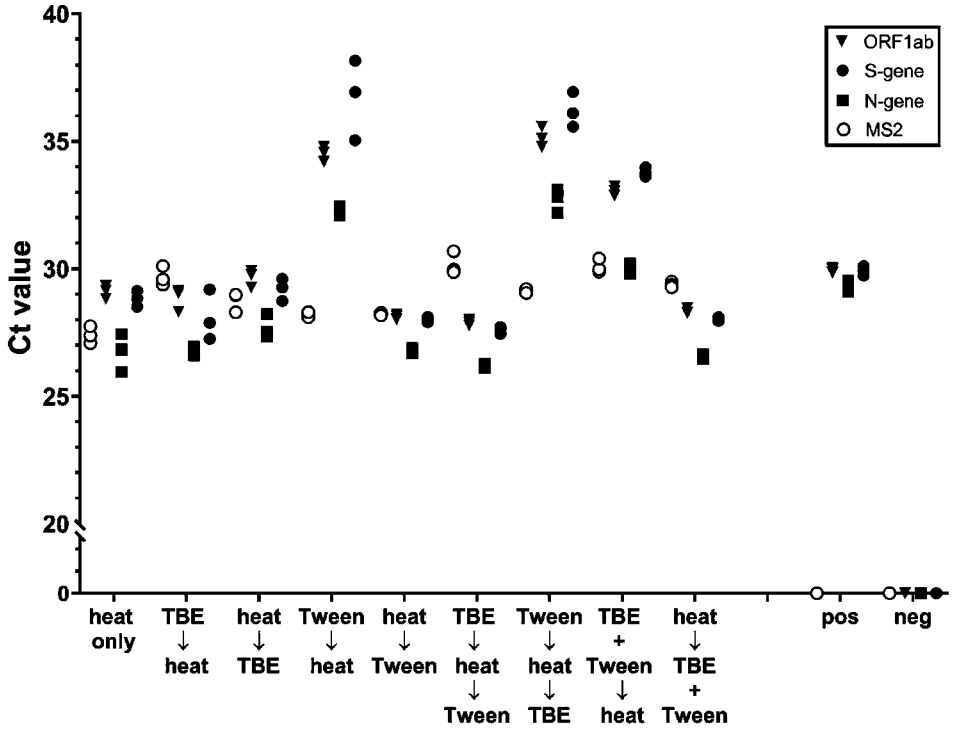
Supporting Figure 1. Saliva collection buffer titration. γ-irradiated SARS-CoV-2 ($1.0x10^3$ (a) or $1.0x10^4$ (b) viral copies/mL) was spiked into fresh human saliva (SARS-CoV-2 negative) and combined with Tris-Borate-EDTA buffer (TBE), Tris-EDTA buffer (TE), or Phosphate Buffered Saline (PBS), at a final working concentration of 2X, 1.5X, 1X, or 0.5X. Samples (0.5 mL in 50 mL conical tubes) were incubated in a hot water bath at 95°C for 30 min. All saliva samples were spiked with purified MS2 bacteriophage (1:40 MS2:sample) as an internal control. Virus-spiked saliva samples, a positive control (pos; SARS-CoV-2 positive control, $5.0x10^3$ copies/mL, no MS2) and a negative control (neg; water, no MS2) were directly analyzed by RT-qPCR, in triplicate, for SARS-CoV-2 ORF1ab (green triangle), N-gene (red square), and S-gene (blue circle), and MS2 (open circle). Undetermined Ct values are plotted at 0.

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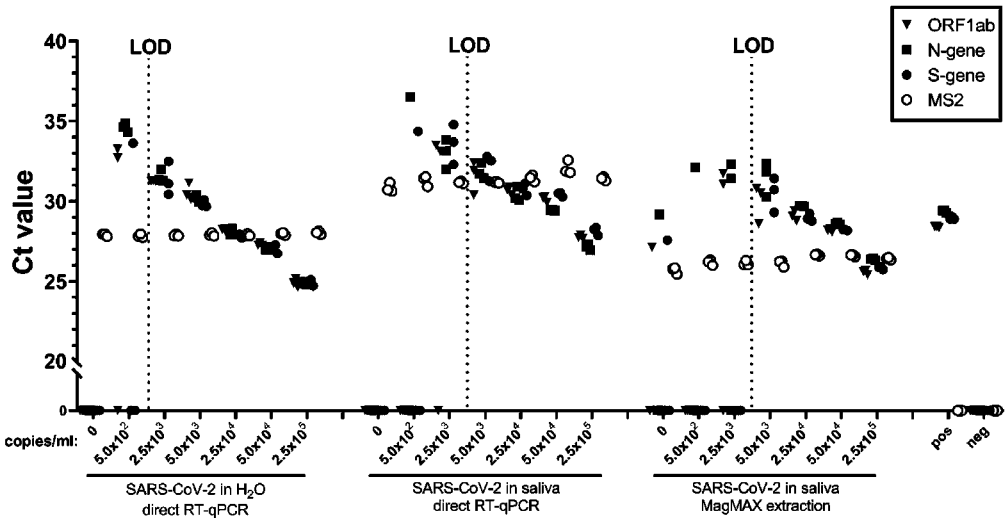
Supporting Figure 2. RNA stabilizing additive optimization. γ-irradiated SARS-CoV-2 ($1.0x10^4$ viral copies/mL) was spiked into fresh human saliva (SARS-CoV-2 negative) and combined with TBE buffer, at a final working concentration of 1X. Samples (0.5 mL in 50 mL conical tubes) were incubated in a hot water bath at 95°C for 30 min. Following heat treatment, virus-spiked saliva was combined with various RNA stabilizing agents, including RNaseI (1 U/µL), carrier RNA (0.05 µg/mL), glycogen (1 µg/µL), TCEP/EDTA (1X), Proteinase K (5 µg/µL), RNase-free BSA (1.25 mg/mL), RNAlater (1:1 ratio in place of TBE), or PBS/DTT (6.5 mM DTT in PBS, diluted 1:1 in place of TBE). All saliva samples were spiked with purified MS2 bacteriophage (1:40 MS2:sample) as an internal control. Virus-spiked saliva samples with or without additives, a positive control (pos; SARS-CoV-2 positive control, $5.0x10^3$ copies/mL, no MS2) and a negative control (neg; water, no MS2) were directly analyzed by RT-qPCR, in triplicate, for SARS-CoV-2 ORF1ab (green triangle), N-gene (red square), and S-gene (blue circle), and MS2 (open circle). Undetermined Ct values are plotted at 0.

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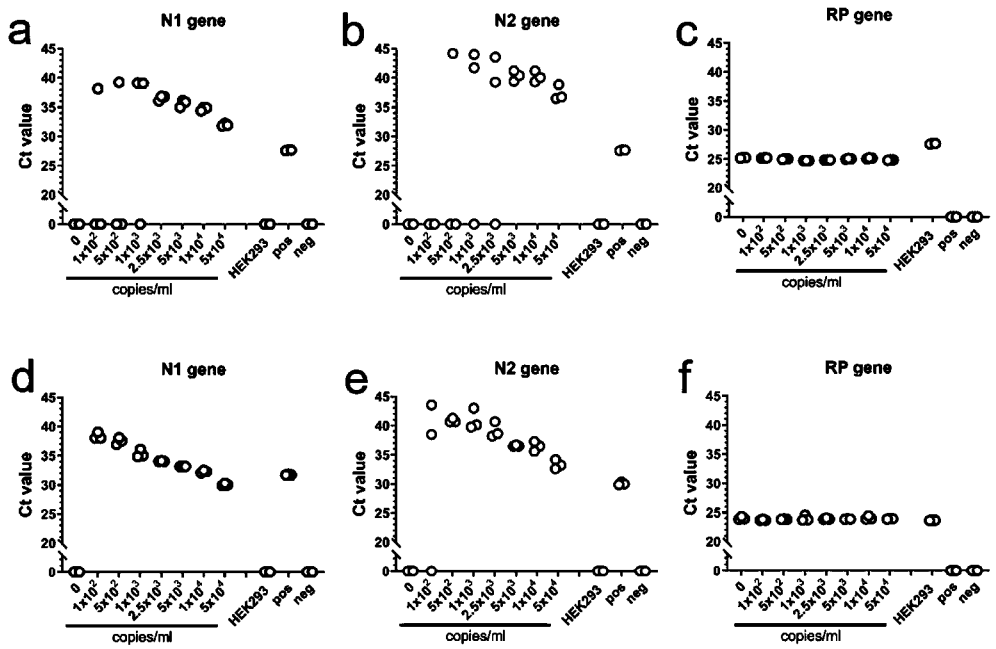
Supporting Figure 3. Workflow of TBE and Tween addition in relation to heat. γ-irradiated SARS-CoV-2 ($1.0x10^5$ viral copies/mL) was spiked into fresh human saliva (SARS-CoV-2 negative) and combined with TBE buffer (1:10, final concentration 1X) and Tween 20 (1:20, final concentration 0.5%) alone or in combination, before or after heat treatment at 95°C for 30 min. All saliva samples were spiked with purified MS2 bacteriophage (1:40 MS2:sample) as an internal control. Virus-spiked saliva samples, a positive control (pos; SARS-CoV-2 positive control, $5.0x10^3$ copies/mL, no MS2) and a negative control (neg; water, no MS2) were directly analyzed by RT-qPCR, in triplicate, for SARS-CoV-2 ORF1ab (green triangle), N-gene (red square), and S-gene (blue circle), and MS2 (open circle). Undetermined Ct values are plotted at 0.

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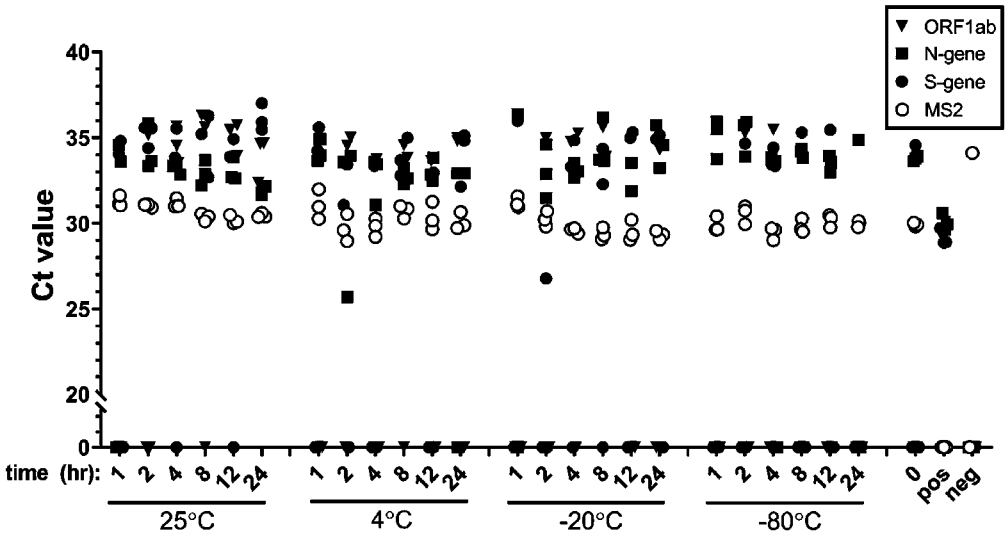
Supporting Figure 4. Limit of detection optimization. Heat-inactivated SARS-CoV-2 was spiked into fresh human saliva (SARS-CoV-2 negative) in 0.5X TE or water at $5.0x10^2$, $2.5x10^3$, $5.0x10^3$, $2.5x10^4$, $5.0x10^4$, and $2.5x10^5$ viral copies/mL. Samples were incubated at 95°C for 30 min. All samples were spiked with purified MS2 bacteriophage (1:40 MS2:sample) as an internal control. Virus-spiked samples were either processed for RNA extraction using a commercially available kit (MagMAX), or directly analyzed by RT-qPCR (direct saliva). All samples, including a positive control (pos; SARS-CoV-2 positive control, $5.0x10^3$ copies/mL, no MS2) and a negative control (neg; water, no MS2) were analyzed by RT-qPCR, in triplicate, for SARS-CoV-2 ORF1ab (green triangle), N-gene (red square), and S-gene (blue circle), and MS2 (open circle). Undetermined Ct values are plotted at 0. The limit of detection (LOD) is indicated by the vertical dotted line.

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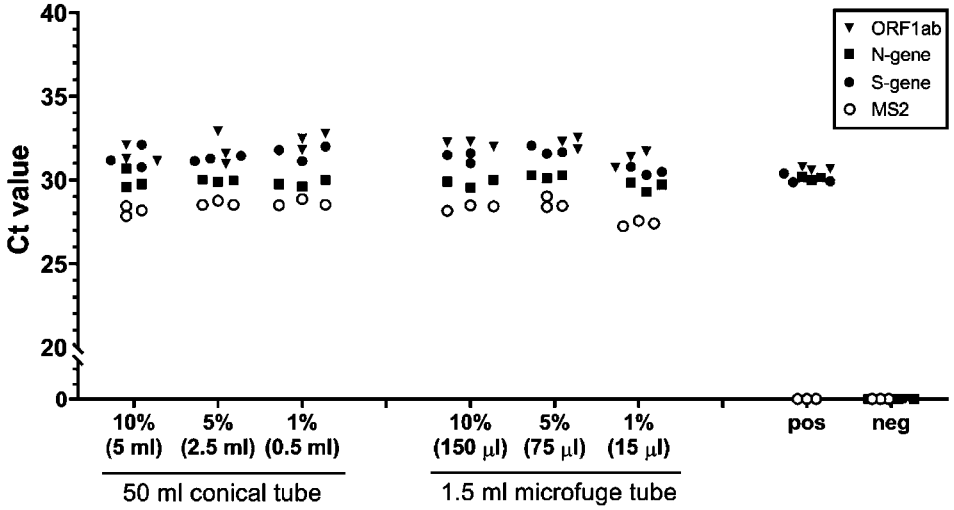
Supporting Figure 5. LOD of direct saliva-to-RT-qPCR SARS-CoV-2 detection using CDC-approved primers and probes. Heat-inactivated (a, b, c) and γ-irradiated (d, e, f) SARS-CoV-2 was spiked into fresh human saliva (SARS-CoV-2 negative) in 1X Tris-Borate-EDTA buffer (TBE) at $1.0x10^2$, $5.0x10^2$, $1.0x10^3$, $2.5x10^3$, $5.0x10^3$, $1.0x10^4$, and $5.0x10^4$ viral copies/mL. Samples were incubated at 95°C for 30 min. Virus-spiked saliva samples, a positive control (pos; SARS-CoV-2 positive control, $5.0x10^3$ copies/mL) and a negative control (neg; water) were directly analyzed by RT-qPCR, in triplicate, for SARS-CoV-2 N1 gene (a, d) and N2 gene (b, e), and the human RP gene (c, f). Undetermined Ct values are plotted at 0.

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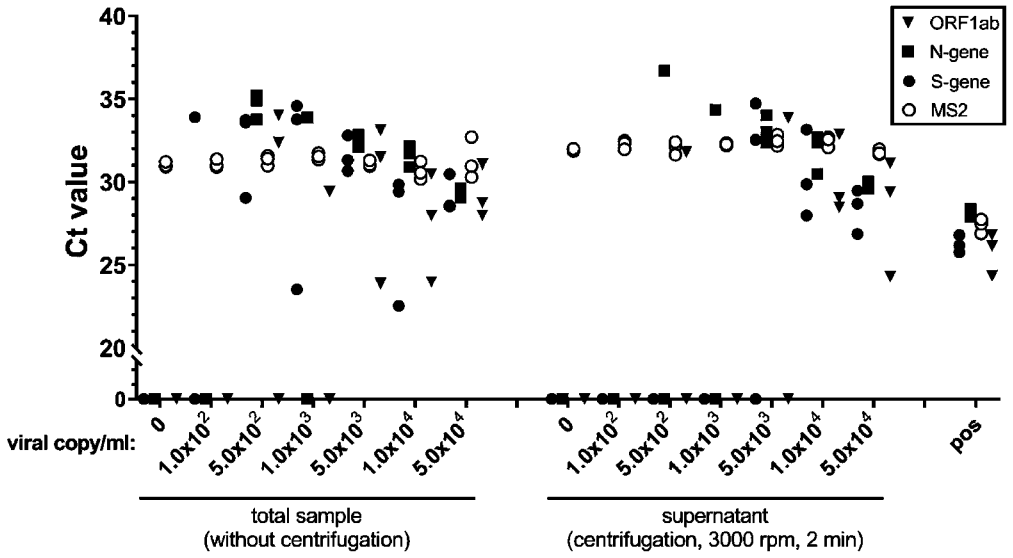
Supporting Figure 6. Stability of saliva samples. γ-irradiated SARS-CoV-2 ($1.0x10^4$ viral copies/mL) was spiked into fresh human saliva (SARS-CoV-2 negative) and combined with TBE buffer 1:1 to a final working concentration of 1X. Samples (0.5 mL in 50 mL conical tubes) were stored at 25°C (ambient temperature), 4°C, -20°C, or -80°C for 1, 2, 4, 8, 12, and 24 hours. Following storage, samples were incubated in a hot water bath at 95°C for 30 min. All saliva samples were spiked with purified MS2 bacteriophage (1:40 MS2:sample) as an internal control. Virus-spiked saliva samples stored under different conditions, a freshly prepared virus-spiked saliva sample (0 hr), a positive control (pos; SARS-CoV-2 positive control, $5.0x10^3$ copies/mL, no MS2) and a negative control (neg; water, no MS2) were directly analyzed by RT-qPCR, in triplicate, for SARS-CoV-2 ORF1ab (green triangle), N-gene (red square), and S-gene (blue circle), and MS2 (open circle). Undetermined Ct values are plotted at 0.

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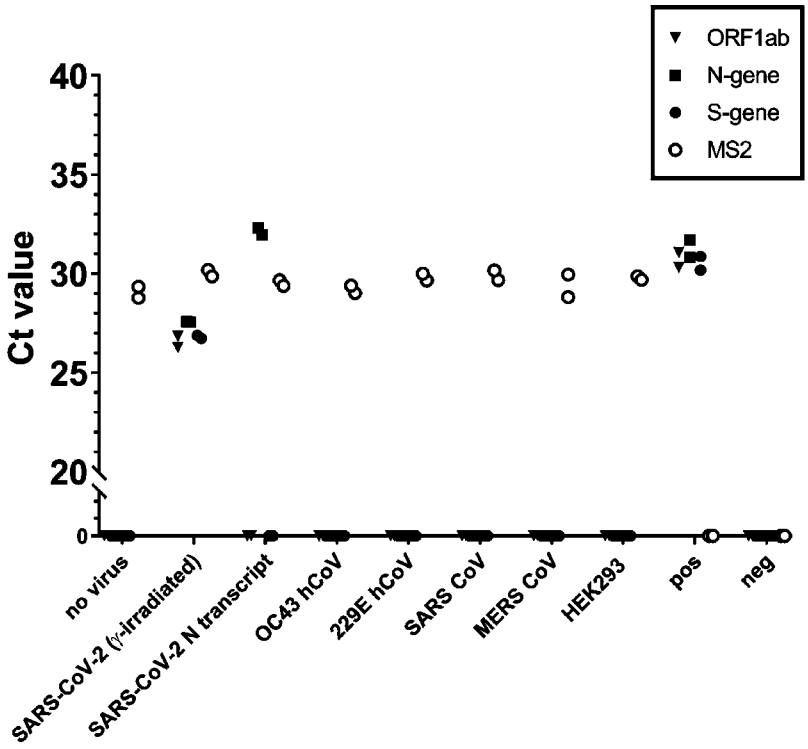
Supporting Figure 7. Effect of sample volume on SARS-CoV-2 detection. γ-irradiated SARS-CoV-2 ($1.0x10^4$ viral copies/mL) was spiked into fresh human saliva (SARS-CoV-2 negative) and combined with TBE buffer 1:1 at a final working concentration of 1X. The sample was distributed into either 50 mL conical or 1.5 mL microfuge tubes, at either 10% (5 mL in 50 mL conical, 150 µL in 1.5 ml microfuge), 5% (2.5 mL in 50 ml conical, 75 µL in 1.5 ml microfuge), or 1% (0.5 mL in 50 mL conical, 15 µL in 1.5 mL microfuge) the vessel storage capacity. Samples were incubated in a hot water bath at 95°C for 30 min. All saliva samples were spiked with purified MS2 bacteriophage (1:40 MS2:sample) as an internal control. Virus-spiked saliva samples, a positive control (pos; SARS-CoV-2 positive control, $5.0x10^3$ copies/mL, no MS2) and a negative control (neg; water, no MS2) were directly analyzed by RT-qPCR, in triplicate, for SARS-CoV-2 ORF1ab (green triangle), N-gene (red square), and S-gene (blue circle), and MS2 (open circle). Undetermined Ct values are plotted at 0.

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Supporting Figure 8. Effect of centrifugation on SARS-CoV-2 detection. Heat-inactivated SARS-CoV-2 ($1.0x10^2$, $5.0x10^2$, $1.0x10^3$, $5.0x10^3$, $1.0x10^4$, and $5.0x10^4$ viral copies/mL) was spiked into fresh human saliva (SARS-CoV-2 negative) and combined with TBE buffer 1:1 at a final working concentration of 1X. Samples were heat treated at 95°C for 30 min, then treated with or without centrifugation at 3000 rpm for 2 min. All saliva samples were spiked with purified MS2 bacteriophage (1:40 MS2:sample) as an internal control. Virus-spiked saliva samples, centrifugation supernatants, a positive control (pos; SARS-CoV-2 positive control, $5.0x10^3$ copies/mL) and a negative control (neg; water) were directly analyzed by RT-qPCR, in triplicate, for SARS-CoV-2 ORF1ab (green triangle), N-gene (red square), and S-gene (blue circle), and MS2 (open circle). Undetermined Ct values are plotted at 0.

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Supporting Figure 9. Specificity of SARS-CoV-2 detection system. Commercially available saliva (Lee Biosciences and Innovative Research) were combined in equal proportions, diluted 1:1 with 2X TBE buffer, and spiked $1.0x10^5$ viral copies/mL of SARS-CoV-2 (γ-irradiated virus or synthetic N-transcript RNA), human coronaviruses (229E, OC43), SARS and MERS synthetic RNA, and human RNA (purified from HEK 293 cells). Samples were heat treated at 95°C for 30 min. All saliva samples were spiked with purified MS2 bacteriophage (1:40 MS2:sample) as an internal control. Virus-spiked saliva samples, a positive control (pos; SARS-CoV-2 positive control, $5.0x10^3$ copies/mL, no MS2) and a negative control (neg; water, no MS2) were directly analyzed by RT-qPCR, in triplicate, for SARS-CoV-2 ORF1ab (green triangle), N-gene (red square), and S-gene (blue circle), and MS2 (open circle). Undetermined Ct values are plotted at 0.

APPENDIX 12

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Emerging Mutations & Variants of SARS-CoV-2
The world leader in serving science
1
Intended use of the products in this presentation vary.
Please refer to the instructions for use for applicable intended use.

SARS-CoV-2 Viral Mutations
Viruses mutate. RNA viruses, like SARS-CoV-2, mutate at high rates in response to selective pressures
• Continued uncontrolled transmission of SARS-CoV-2 in many parts of the world is creating conditions for significant virus evolution
• Throughout the pandemic, SARS-CoV-2 has been mutating at a rate of about one to two mutations per month
• Some recently identified variants, however, have acquired mutations much more rapidly than scientists expect
Thermo Fisher SCIENTIFIC

Potential Implications of New SARS-CoV-2 Variants

What might new SARS-CoV-2 mutations and variants mean?

- Ability to spread more quickly in humans
- Ability to cause either milder or more severe disease in humans
- Decreased susceptibility to therapeutic agents such as monoclonal antibodies
- Ability to evade vaccine-induced immunity
- **Ability to evade detection by specific diagnostic tests**

Source: US CDC https://www.cdc.gov/coronavirus/2019-ncov/more/science-and-research/scientific-brief-emerging-variants.html

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TaqPath COVID-19 assay designs:
Built With Mutations In Mind
IVD
For in vitro diagnostic use.
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Thermo Fisher's Approach to SARS-CoV-2 Assay Design
Informed by experience in global public health response
ThermoFisher SCIENTIFIC
GISAID
NCBI
Genomic Conservation
Specificity
Redundancy
Utilizes conserved* areas of genome
Targets regions unique* to SARS-CoV-2
Highly effective for targeting SARS-CoV-2
*Based on Global Initiative on Sharing Avian Influenza Data (GISAID) and GenBank databases. Mapped to >55,000 complete genomes as of December 9, 2020
*Due to extreme interest in this emergency, GISAID Genomes are being frequently updated and other public databases. Thermo Fisher regularly monitors public genomic databases to maintain a record of SARS-CoV-2 mutations.
5

Thermo Fisher SCIENTIFIC
Superior Targeting for Specificity to SARS-CoV-2
TaqPath COVID-19 CE-IVD RT-PCR Kit targets 3 areas of SARS-CoV-2 virus
3 targeted regions
TaqPath COVID-19 assay
TaqPath COVID-19 assay
Taq-Path
ORF1a
ORF1b
S
3
M
N
E
7
10b
8
• Targets areas unique to SARS-CoV-2* virus to reduce detection of other coronaviruses
• Multiple targeted regions compensate for virus mutations
100% specific to currently available SARS-CoV-2 complete genomes*
*GISAID and GenBank databases as of December 9, 2020
IVD For In Vitro Diagnostic Use
6

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TaqPath COVID-19 assay designs:
Built With Mutations In Mind
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Thermo Fisher's Approach to SARS-CoV-2 Assay Design
Informed by experience in global public health response
ThermoFisher SCIENTIFIC
GISAID
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Genomic Conservation
Specificity
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Utilizes conserved* areas of genome
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8

Thermo Fisher SCIENTIFIC
Superior Targeting for Specificity to SARS-CoV-2
TaqPath COVID-19 Combo Kit and Advanced Kit target 3 areas of SARS-CoV-2 virus
3 targeted regions
TaqPath COVID-19 assay
TaqPath COVID-19 assay
Taq-Path
ORF1a
ORF1b
S
3
M
N
E
7
10b
8
Targets areas unique to SARS-CoV-2* virus to reduce detection of other coronaviruses
Multiple targeted regions compensate for virus mutations
100% specific to currently available SARS-CoV-2 complete genomes*
*GISAID and GenBank databases as of December 9, 2020
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B.1.1.7 (UK) Variant, 69-70del mutation, and S gene dropout
IVD For in vitro diagnostic use.
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New SARS-CoV-2 UK Variant: B.1.1.7 (or VOC-202012/01)

Thermo Fisher SCIENTIFIC

Emerged in September 2020, reported widely in December 2020, now reported in 30+ countries*

- Acquired mutations much more quickly than expected—17 in total
  - 8 S-gene mutations including one *69-70del* mutation which leads to the loss of two amino acids in the spike protein
  - Estimated 70% more transmissible**
  - No evidence of increased disease severity**
  - Highly likely that COVID-19 vaccines remain effective against this variant***

69-70del is ***not*** exclusive to B.1.1.7

Concerns

- Increased transmission rates may stress at-capacity healthcare systems
- Questions about how mutations affect sensitivity claims of diagnostic tests

*As of December 30, 2020

**European Centre for Disease Prevention and Control 20 Dec 2020 Threat Assessment Brief

***https://www.npr.org/sections/coronavirus-live-updates/2020/12/22/949150817/biontech-ceo-says-highly-likely-vaccine-is-effective-against-u-k-virus-variant

Effect of B.1.1.7 Variant on TaqPath COVID-19 Kits' Claims

ThermoFisher SCIENTIFIC

Implications of multi-target assay design on emerging mutations

- One mutation out of all 17 in B.1.1.7 is important to be aware of in relation to our kits—*69-70del* mutation
- *69-70del* has **not been found to generate false negative test results when using any of our kits**
  - Strategic value of utilizing multi-target assay designs
- Specific to TaqPath COVID-19 CE-IVD RT-PCR Kit
  - Samples with *69-70del* will show an S gene "drop out" (i.e., the S gene will not be detected)
  - This drop out does ***not*** mean a result is negative, only that the S gene could not be detected
  - Interpretive software requires only 2 of 3 targets detected for result to be called 'positive'

All listed products meet sensitivity and specificity claims with B.1.1.7 variant

Kit name	SARS-CoV-2 targets	S gene drop out?	SKU
TaqPath COVID-19 CE-IVD RT-PCR Kit	S, N, *orf1ab* (3 separate channels)	**Yes**	A48067
TaqPath COVID-19, Flu A/B, RSV Combo Kit	S, N (same channel)	No	A49867

S gene drop out can signal the presence of *69-70del* and, potentially, the B.1.1.7 variant

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How S gene Drop Out Can Signal a B.1.1.7 Specimen

ThermoFisher SCIENTIFIC

TaqPath COVID-19 CE-IVD RT-PCR Kit can signal the presence of *69-70del* and, potentially, the B.1.1.7 variant

69-70del is ***not*** exclusive to B.1.1.7

- Public Health England (PHE) reported that the *60-70del* was present in >99% of sequenced samples with S gene drop out
- European CDC recommends a multi-target PCR assays that include an S gene target affected by the deletion as it can signal the presence of *69-70del* mutation for further investigation*
  - **The TaqPath COVID-19 CE-IVD RT-PCR Kit is such an assay and can be useful to support surveillance and epidemiology efforts****
- Confirmation of the *69-70del* and B.1.1.7 variant can be accomplished by sequencing
  - Sanger sequencing
  - Next-generation sequencing (NGS)

13 CE IVD For In Vitro Diagnostic Use

*European Centre for Disease Prevention and Control 20 Dec 2020 Threat Assessment Brief

**https://www.sciencemag.org/news/2020/12/mutant-coronavirus-united-kingdom-sets-alarms-its-importance-remains-unclear

What about our SARS-CoV-2 Detection (RUO) Kits?

Assay sensitivity and specificity performance is **not** affected by the B.1.1.7 variant

Kit name	SARS-CoV-2 targets	S gene drop out?	SKU
TaqMan 2019-nCoV Assay Kit v1 (singleplex)	S, N (same channel)	No	A47532
TaqMan SARS-CoV-2, Flu A, Flu B RT-PCR Assay Kit	S, N (same channel)	No	A47701
TaqMan SARS-CoV-2, Flu A/B, RSV RT-PCR Assay Kit	S, N (same channel)	No	A47702
TaqCheck SARS-CoV-2 Fast PCR Assay	S, N (same channel)	No	A47693

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B.1.1.7 (UK) Variant, 69-70del mutation, and S gene dropout
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New SARS-CoV-2 UK Variant: B.1.1.7 (or VOC-202012/01)

Thermo Fisher SCIENTIFIC

Emerged in September 2020, reported widely in December 2020, Now reported in 30+ countries*

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  - 8 S-gene mutations including one *69-70del* mutation which leads to the loss of two amino acids in the spike protein
  - Estimated 70% more transmissible**
  - No evidence of increased disease severity**
  - Highly likely that COVID-19 vaccines remain effective against this variant***

69-70del is ***not*** exclusive to B.1.1.7

Concerns

- Increased transmission rates may stress at-capacity healthcare systems
- Questions about how mutations affect sensitivity claims of diagnostic tests

*As of December 30, 2020

**European Centre for Disease Prevention and Control 20 Dec 2020 Threat Assessment Brief

***https://www.npr.org/sections/coronavirus-live-updates/2020/12/22/949150817/biontech-ceo-says-highly-likely-vaccine-is-effective-against-u-k-virus-variant

Effect of B.1.1.7 Variant on TaqPath COVID-19 Kits' Claims

Implications of Multi-Target Assay Design on Emerging Mutations

- One mutation out of all 17 in B.1.1.7 is important to be aware of in relation to our kits—*69-70del* mutation
- *69-70del* has **not been found to generate false negative test results when using any of our kits**
  - Strategic value of utilizing multi-target assay designs
  - Specific to TaqPath COVID-19 Combo Kit & Advanced
    - Samples with *69-70del* show an S gene "drop out" (i.e., the S gene will not be detected)
    - Drop out does ***not*** mean a result is negative, only that the S gene could not be detected
    - Interpretive software requires only 2 of 3 targets detected for result to be called 'positive'

All listed products meet sensitivity and specificity claims with B.1.1.7 variant

Kit name	SARS-CoV-2 targets	S gene drop out?	SKU
TaqPath COVID-19 Combo Kit	S, N, *orf1ab* (3 separate channels)	**Yes**	A47814
TaqPath COVID-19 Combo Kit Advanced	S, N (same channel)	**Yes**	A47813

S gene drop out can signal the presence of *69-70del* and, potentially, the B.1.1.7 variant

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How S gene Drop Out Can Signal a B.1.1.7 Specimen

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TaqPath COVID-19 Combo Kit can signal the presence of *69-70del* and, potentially, the B.1.1.7 variant

69-70del is ***not*** exclusive to B.1.1.7

- Public Health England (PHE) reported that the *60-70del* was present in >99% of sequenced samples with S gene drop out
- European CDC recommends a multi-target PCR assays that include an S gene target affected by the deletion as it can signal the presence of *69-70del* mutation for further investigation*
- **The TaqPath COVID-19 Combo Kit is such an assay and can be useful to support surveillance and epidemiology efforts****
- Confirmation of the *69-70del* and B.1.1.7 variant can be accomplished by sequencing
- Sanger sequencing
- Next-generation sequencing (NGS)

*European Centre for Disease Prevention and Control 20 Dec 2020 Threat Assessment Brief
**https://www.sciencemag.org/news/2020/12/mutant-coronavirus-united-kingdom-sets-alarms-its-importance-remains-unclear

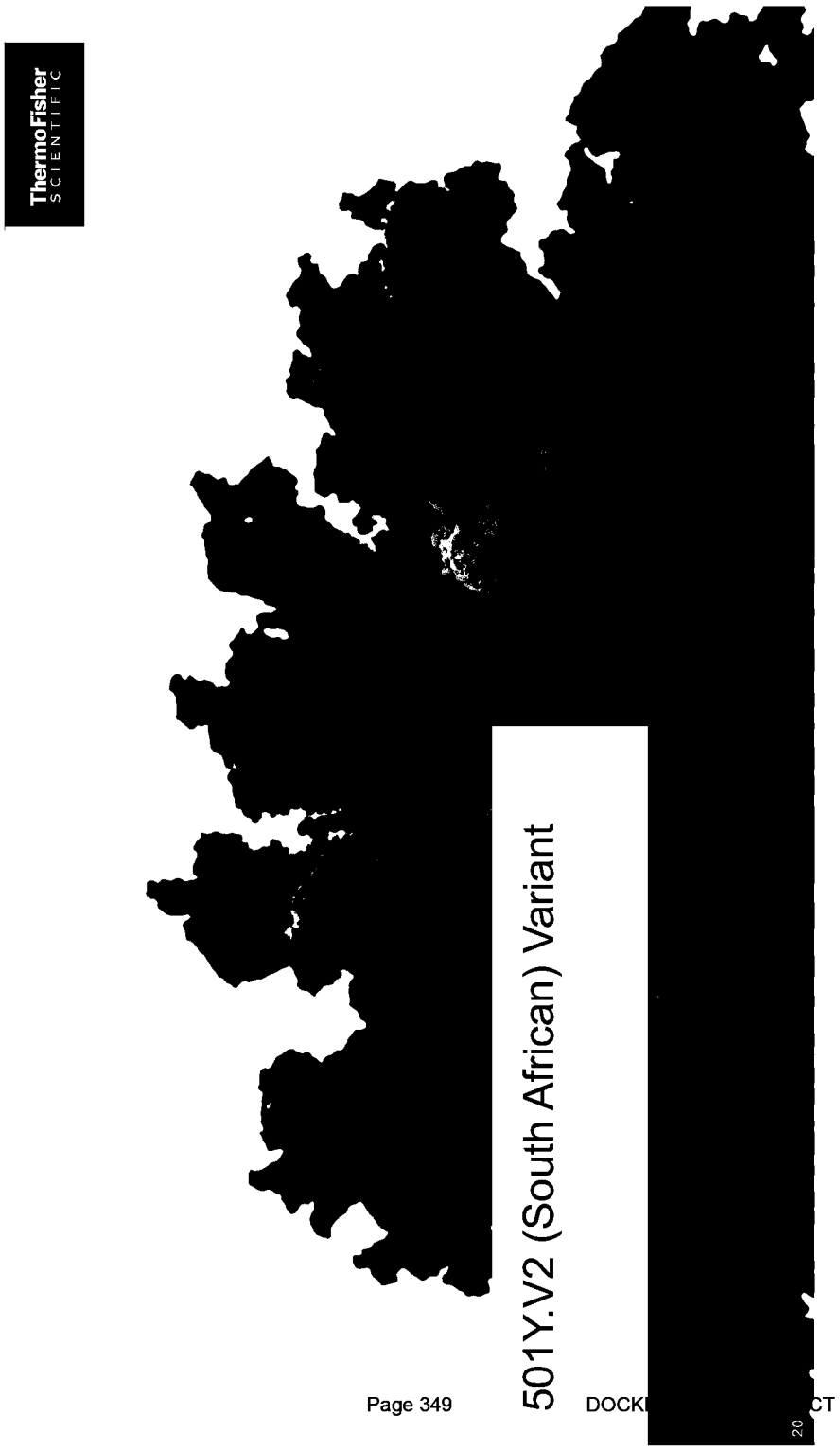
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Assay sensitivity and specificity performance is **not** affected by the B.1.1.7 variant

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TaqMan 2019-nCoV Assay Kit v1 (singleplex)	S, N (same channel)	No	A47532
TaqMan SARS-CoV-2, Flu A, Flu B RT-PCR Assay Kit	S, N (same channel)	No	A47701
TaqMan SARS-CoV-2, Flu A/B, RSV RT-PCR Assay Kit	S, N (same channel)	No	A47702
TaqCheck SARS-CoV-2 Fast PCR Assay	S, N (same channel)	No	A47693

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501Y.V2 (South African) Variant
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New SARS-CoV-2 South African Variant: 501Y.V2

First reported in South Africa in December 2020. Now in Austria, Norway, Japan, France…

- Reportedly more contagious but no evidence of increased disease severity
- Shares N501Y mutation with B.1.1.7 (UK variant) but emerged independently and is phylogenetically different

Concerns:

- Linked to higher viral load & increased transmission which could stress at-capacity healthcare systems
- May be associated with poor response to antibody-based therapies*
- Worries about current vaccine effectiveness due to multiple mutations (N501Y, E484K, K417H) in receptor binding domain (RBD)

* https://www.cnbc.com/2021/01/05/south-africa-covid-variant-appears-to-obviate-antibody-drugs-dr-scott-gottlieb-says.html

Effect of 501Y.V2 Variant on TaqPath COVID-19 Kits' Claims

Thermo Fisher SCIENTIFIC

To date, the mutations observed in the 501Y.V2 variant have ***NOT*** been found by Thermo Fisher, the US FDA, or its customers to impact test results obtained with our TaqPath COVID-19 portfolio assays.

- No impact on our TaqPath COVID-19 portfolio of assays
  - Neither N501Y mutation nor any others in 501Y.V2 have been found to have any impact on our assays
  - Does not have 69-70del and therefore we **will not see S-gene dropout**
- Confirmation of the 501Y.V2 variant and mutations can be accomplished by sequencing:
  - Sanger sequencing
  - Next-generation sequencing (NGS)

Intended use of the products on this slide vary. Please refer to the instructions for use for applicable intended use.

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Sequencing tools for SARS-CoV-2 mutation and variant surveillance
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Epidemiological Surveillance to Monitor Mutations

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Epidemiological surveillance is conducted to ensure viral diseases match the reference strain and to monitor possible mutations, since any changes in the viral genome can impact public health policies and options, how the illness spreads in the population, potential study of treatment options, and vaccine development research.

There are still many unknows about SARS-CoV-2 which need to be understood to better predict virus strain evolution:

- How can we detect new variants?
- What is the origin of a new variant and how prevalent is it?
- Will current molecular or antigen-based tests miss detecting a variant?
- Does a variant spread more quickly?
- Does a variant lead to higher disease severity?
- What is the vaccine efficacy against a new variant?

Thermo Fisher Sequencing Portfolio: Surveillance and Confirmation of Emerging Variants

Thermo Fisher SCIENTIFIC

- Value of sequencing for surveillance of SARS-CoV-2 and variant confirmation
  - Confirm the presence of known or emerging mutations
  - Use molecular fingerprints to assign people to genetic clusters and build more definitive transmission chains
  - Help understand how the virus is spreading within local communities, across a nation, and globally
- **NGS**: amplify full viral genome for viral genetic surveillance and confirmation of many mutations
  - Amplify the full viral genome creating a more robust picture of potential transmission patterns and clusters
  - Especially important for considering disease control strategies and mitigating emerging hot spots
- **Sanger sequencing**: targeted sequencing for single samples and confirmation of few mutations
  - Single sample, whole-genome Sanger sequencing to identify mutations and to confirm data from NGS
  - Targeted sequencing of short stretches of the viral genome to confirm know variants

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Ion AmpliSeq SARS-CoV-2 Research Panel

One assay surveying complete SARS-CoV-2 genome for epidemiological investigation and mutation detection

Pool	No. Total Amplicons
1	247 (242 unique)*
2	

- Amplicon length range: 125-275 bp
- 237 amplicons specific to SARS-CoV-2 + 5 human expression controls
- >99% coverage of SARS-CoV-2 genome (~30kb)
- All potential serotypes covered

Intelligent design provides exceptional protection against naturally occurring variation and ensures robust performance even as the virus rapidly mutates

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Sanger Sequencing Confirmation of S Gene Mutations and B.1.1.7 variant
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TaqPath workflow
Receive samples
Prepare samples
Prepare and run RT-qPCR
Analyze and report
(+) sample: Is S-gene detected?
Yes
No = Reflex to Sanger sequencing
Report results
Sanger sequencing confirmation workflow
Prepared sample
S-gene amplification
Sanger Sequencing
Variant Analysis
• Samples initially processed using TaqPath COVID-19 assay kits
- If the sample is positive **and** the S-gene is undetectable (S-gene "dropout") the sample can be reflex-tested by Sanger sequencing
• Use samples prepared in initial workflow to conduct Sanger sequencing for:
- Confirmation of 69-70del mutation (link to protocol)
- Subtype verification of B.1.1.7 viral strain
27 Intended use of the products on this slide vary. Please refer to the instructions for use for applicable intended use.

APPENDIX 13

applied biosystems INSTRUCTIONS FOR USE

TaqPath™ COVID-19 Combo Kit

Multiplex real-time RT-PCR test intended for the presumptive qualitative detection of nucleic acid from SARS-CoV-2

Catalog Numbers A47813 and A47814

Pub. No. MAN0019181 Rev. A.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

- Intended Use . . . 2
- Product description . . . 2
- Contents and storage . . . 2
- Required materials not supplied . . . 3
- Warnings and precautions . . . 4
- Samples and controls . . . 4
- Workflow . . . 4
- Extract RNA with the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit . . . 5
- Perform RT-PCR . . . 7
- Analyze the data . . . 9
- Results interpretation for patient samples . . . 9
- Assay limitations . . . 9
- Conditions of authorization for labs . . . 10
- Performance characteristics . . . 11
- Related documentation . . . 14
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IVD

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ThermoFisher

Intended Use

TaqPath™ COVID-19 Combo Kit contains the assays and controls for a real-time reverse transcription polymerase chain reaction (RT-PCR) test intended for the presumptive qualitative detection of nucleic acid from SARS-CoV-2 in nasopharyngeal swab, nasopharyngeal aspirate, and bronchoalveolar lavage (BAL) specimens from individuals meeting CDC COVID-19 clinical criteria (e.g., clinical signs and symptoms associated with SARS-CoV-2 infection) in conjunction with CDC COVID-19 epidemiological criteria (e.g., history of residence in or travel to a geographic region with active SARS-CoV-2 transmission at the time of travel, or other epidemiologic criteria for which SARS-CoV-2 testing may be indicated).

Testing is limited to laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, to perform high complexity tests, or by similarly qualified non-U.S. laboratories.

Results are for the identification of SARS-CoV-2 RNA. The SARS-CoV-2 RNA is generally detectable in nasopharyngeal swab, nasopharyngeal aspirate, and bronchoalveolar lavage (BAL) specimens during the acute phase of infection. Positive results are indicative of active infection but do not rule out bacterial infection or co-infection with other viruses. The agent detected may not be the definite cause of disease. Laboratories within the United States and its territories are required to report all positive results to the appropriate public health authorities.

Negative results do not preclude SARS-CoV-2 infection and should not be used as the sole basis for patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

Testing with the TaqPath™ COVID-19 Combo Kit is intended for use by trained clinical laboratory personnel specifically instructed and trained in the techniques of real-time PCR and *in vitro* diagnostic procedures. The TaqPath™ COVID-19 Combo Kit is only for use under the Food and Drug Administration's Emergency Use Authorization.

Product description

The TaqPath™ RT-PCR COVID-19 Kit, packaged as part of the TaqPath™ COVID-19 Combo Kit, includes the assays and controls for a multiplex real-time RT-PCR test for the presumptive qualitative detection of RNA from SARS-CoV-2 in nasopharyngeal swab, nasopharyngeal aspirate, and bronchoalveolar lavage (BAL) specimens from patients who meet CDC COVID-19 clinical criteria in conjunction with CDC COVID-19 epidemiological criteria. TaqPath™ COVID-19 Combo Kit includes the following components:

- TaqPath™ RT-PCR COVID-19 Kit
  - COVID-19 Real Time PCR Assay Multiplex—Multiplexed assays that contain three primer/probe sets specific to different SARS-CoV-2 genomic regions and primers/probes for bacteriophage MS2
  - MS2 Phage Control—Internal process control for nucleic acid extraction
- TaqPath™ COVID-19 Control—RNA control that contains targets specific to the SARS-CoV-2 genomic regions targeted by the assays

Contents and storage

Table 1 TaqPath™ COVID-19 Combo Kit (Cat. No. A47813)

Component	Description	Amount	Storage
TaqPath™ RT-PCR COVID-19 Kit, 100 reactions (Cat. No. A47815)[1]	COVID-19 Real Time PCR Assay Multiplex (Gene Orf-1ab, N Protein, S Protein, MS2)	150 µL	–30°C to –10°C
	MS2 Phage Control	2 × 500 µL	–30°C to –10°C
TaqPath™ COVID-19 Control Kit (Cat. No. A47816)[1]	TaqPath™ COVID-19 Control (1 x 10^4 copies/µL)	2 × 10 µL	≤ –70°C
	TaqPath™ COVID-19 Control Dilution Buffer	2 × 250 µL	–30°C to –10°C

[1] This kit can be ordered as a stand-alone kit.

Table 2 TaqPath™ COVID-19 Combo Kit, 1,000 reactions (Cat. No. A47814)

Component	Description	Amount	Storage
TaqPath™ RT-PCR COVID-19 Kit, 1,000 reactions (Cat. No. A47817)[1]	COVID-19 Real Time PCR Assay Multiplex (Gene Orf-1ab, N Protein, S Protein, MS2)	1,500 µL	–30°C to –10°C
	MS2 Phage Control	20 × 500 µL	–30°C to –10°C
TaqPath™ COVID-19 Control Kit (Cat. No. A47816)[1]	TaqPath™ COVID-19 Control (1 x 10^4 copies/µL)	2 × 10 µL per kit; 5 kits included	≤ –70°C
	TaqPath™ COVID-19 Control Dilution Buffer	2 × 250 µL per kit; 5 kits included	–30°C to –10°C

[1] This kit can be ordered as a stand-alone kit.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Item	Source
Real-time PCR instrument and equipment	
Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument (used with SDS Software v1.4.1)	4406984 (with laptop computer) 4406985 (with tower computer)
Laboratory freezers • –30°C to –10°C • ≤ –70°C	MLS
Centrifuge, with a rotor for microplates	MLS
Microcentrifuge	MLS
Laboratory mixer, Vortex or equivalent	MLS
Single and multichannel adjustable pipettors (1.00 µL to 1,000.0 µL)	MLS
Cold block or ice	MLS
Automated nucleic acid extraction system and materials	
KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head	5400630
KingFisher™ Flex 96 Deep-Well Heating Block	24075430
KingFisher™ Deepwell 96 Plate	95040450
KingFisher™ 96 KF microplate	97002540
KingFisher™ 96 tip comb for DW magnets	97002534
Kits and reagents	
MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit	A42352 (100 preparations) Also available for 1,000 preparations
TaqPath™ 1-Step Multiplex Master Mix (No ROX™)	A28521, A28522, A28523
100% ethanol, ACS reagent grade or equivalent	MLS
Nuclease-free Water (not DEPC-Treated)	MLS
Tubes, plates, and other consumables	
ABY™ Dye Spectral Calibration Plate for Multiplex qPCR, Fast 96-well	A24734
JUN™ Dye Spectral Calibration Plate for Mutliplex qPCR, Fast 96-well	A24735
MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL	4346906, 4366932
MicroAmp™ Clear Adhesive Film	4306311
MicroAmp™ Optical Adhesive Film	4311971 and 4360954
MicroAmp™ Adhesive Film Applicator	4333183
Nonstick, RNase-free microcentrifuge tubes (1.5 mL and 2.0 mL)	thermofisher.com/plastics
Sterilize aerosol barrier (filtered) pipette tips	thermofisher.com/pipettetips
Data analysis software	
Applied Biosystems™ COVID-19 Interpretive Software v1.0[1]	InstrumentServices@thermofisher.com *or* 1 800 955 6288 (Select option 3, then option 1)

[1] For software installation instructions, see *COVID-19 Interpretive Software Installation Quick Reference* (Pub. No. MAN0019184).

Warnings and precautions

The TaqPath™ RT-PCR COVID-19 Kit workflow should be performed by qualified and trained staff to avoid the risk of erroneous results. Use separate areas for the preparation of patient samples and controls to prevent false positive results. Samples and reagents must be handled under a laminar airflow hood or biological safety cabinet.

- The assay is for *in vitro* diagnostic use under the FDA Emergency Use Authorization Only.
- Specimens should always be treated as if infectious and/or biohazardous in accordance with safe laboratory procedures.
- Follow necessary precautions when handling specimens. Use personal protective equipment (PPE) consistent with current guidelines for the handling of potentially infectious samples.
- Always use pipette tips with aerosol barriers. Tips that are used must be sterile and free from DNases and RNases.
- Do not eat, drink, smoke, or apply cosmetic products in the work areas.
- Modifications to assay reagents, assay protocol, or instrumentation are not permitted, and are in violation of the product Emergency Use Authorization.
- Do not use the kit after the indicated expiry date.
- Dispose of waste in compliance with the local, state, and federal regulations.
- Safety Data Sheets are available upon request.

Samples and controls

Patient samples must be collected according to appropriate laboratory guidelines. Positive and negative test controls must be included to accurately interpret patient test results.

Include the following controls:

Control	Used to monitor	Assays
Positive Control (TaqPath™ COVID-19 Control Kit)	RT-PCR reaction setup and reagent integrity	All three SARS-CoV-2 assays
MS2 Phage Control	RNA extraction	MS2 assay
Negative Control	Cross-contamination during RNA extraction and reaction setup	All three SARS-CoV-2 assays
		MS2 assay

Workflow

Extract RNA from patient sample using the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit

▼

Perform RT-PCR using the Applied Biosystems™ 7500 Fast Dx Real-Time PCR instrument

▼

Analyze data using the Applied Biosystems™ COVID-19 Interpretive Software

▼

Review results interpretation for patient samples

The workflow begins with nucleic acid extraction from nasopharyngeal swab, nasopharyngeal aspirate, or bronchoalveolar lavage (BAL) specimens which arrive in the testing site in Universal Viral Transport Media (VTM). Nucleic acids are isolated and purified from the specimens using the KingFisher™ Flex Purification System (KingFisher) and the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit.

Instructions for extracting RNA using the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit are found in the *MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (automated extraction) User Guide* (Pub. No. MAN0018073).

The purified nucleic acid is reverse transcribed into cDNA and amplified using the TaqPath™ RT-PCR COVID-19 Kit and the Applied Biosystems™ 7500 Fast Dx Real-Time PCR instrument. In the process, the probes anneal to three (3) specific target sequences located between three (3) unique forward and reverse primers for the following genes:

- ORF1ab
- N Protein
- S Protein

During the extension phase of the PCR cycle, the 5' nuclease activity of Taq polymerase degrades the probe, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. With each cycle, additional reporter dye molecules are cleaved from their respective probes, increasing the fluorescence intensity. Fluorescence intensity is monitored at each PCR cycle by the Applied Biosystems™ 7500 Fast Dx Real-Time PCR instrument.

The data are analyzed, then interpreted by the Applied Biosystems™ COVID-19 Interpretive Software.

Extract RNA with the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit

Before you begin

- Determine the number of required reactions based on the number patient samples to be processed, plus one Negative Control per plate.
- Prepare fresh 80% Ethanol using 100% absolute Ethanol and Nuclease-free Water (not DEPC-Treated), sufficient for 1.5 mL per reaction, plus 10% overage.
- Label each KingFisher™ Deepwell 96 Plate (5):

Label	Number of plates
Sample plate	1
Wash 1	1
Wash 2	1
Wash 3	1
Elution plate	1

- Label the KingFisher™ 96 KF microplate (1):

Label	Number of plates
Tip comb	1

- Mark the Negative Control well on the plate.

Set up the instrument

1. Ensure that the KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head is set up with the KingFisher™ Flex 96 Deep-Well Heating Block.

   IMPORTANT! Failure to use the proper magnetic head and heat block results in lower yields and potential harm to the instrument.

2. Ensure that the proper program (**MVP_Flex**) has been downloaded from the product page and loaded onto the instrument.

Prepare the processing plates

Prepare the processing plates according to the following table. Cover the plates with a temporary seal, then store at room temperature for up to 1 hour while you set up the sample plate.

Plate ID	Plate position	Plate type	Reagent	Volume per well
Wash 1 Plate	2	KingFisher™ Deepwell 96 Plate	Wash Buffer	1,000 µL
Wash 2 Plate	3		80% Ethanol	1,000 µL
Wash 3 Plate	4		80% Ethanol	500 µL
Elution Plate	5		Elution Solution	50 µL
Tip Comb	6	Place a KingFisher™ 96 tip comb for DW magnets in a KingFisher™ 96 KF microplate		

Prepare Binding Bead Mix

Prepare the required amount of Binding Bead Mix on each day of use.

1. Vortex the Total Nucleic Acid Magnetic Beads to ensure that the bead mixture is homogenous.
2. For the number of required reactions, prepare the Binding Bead Mix according to the following table:

Component	Volume per well[1]
Binding Solution	530 µL
Total Nucleic Acid Magnetic Beads	20 µL
Total volume per well	550 µL

[1] Include 10% overage when making the Binding Bead Mix for use with multiple reactions.

3. Mix well by inversion, then store at room temperature.

Prepare sample plate

1. Add 10 µL of Proteinase K to each well in the KingFisher™ Deepwell 96 Plate labeled "Sample Plate".
2. Add samples and controls to the appropriate wells:

Component	Instructions
Sample	Add 400 µL of sample to a well.
Negative Control	Add 400 µL of Nuclease-free Water (not DEPC-Treated) to the Negative Control well.

3. Invert the Binding Bead Mix five times gently to mix, then add 550 µL to each sample well and the Negative Control well in the Sample Plate.

   Note: Remix the Binding Bead Mix by inversion frequently during pipetting to ensure even distribution of beads to all samples or wells. The mixture containing the Binding Beads is viscous. Therefore, pipet slowly to ensure that the correct amount is added. DO NOT reuse pipette tips to add Binding Bead Mix to the samples, as the high viscosity will cause variations in volume added.

4. Add 10 µL of MS2 Phage Control to each sample well and to the Negative Control well.

Process the samples

1. Select the program **MVP_Flex** on the KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head.
2. Start the run, then load the prepared plates into position when prompted by the instrument.
3. After the run is complete (~25 minutes after start), immediately remove the Elution Plate from the instrument, then cover the plate.

Note: Significant bead carry over may adversely impact RT-PCR performance.

Store the Elution Plate on ice for immediate use in real-time RT-PCR or seal the plate and store at –20°C for up to 1 month, or at ≤ 70°C for long-term storage.

Perform RT-PCR

Guidelines for RT-PCR with the TaqPath™ RT-PCR COVID-19 Kit

IMPORTANT!

- To prevent contamination, prepare reagents in a PCR workstation or equivalent amplicon-free area. Do not use the same pipette for controls and samples, and always use aerosol barrier pipette tips.
- Maintain an RNase-free environment.
- Protect assays from light.
- Keep samples and components on ice during use.
- Prepare the run plate on ice.
- Run the plate within two hours of preparation.
- Include one Positive Control and one Negative Control on each plate.

Prepare the RT-PCR reactions

1. If frozen, thaw the purified nucleic acid samples and reagents on ice.
2. Gently vortex the samples and reagents, then centrifuge briefly to collect liquid at the bottom of the tube or sample plate.
3. **Prepare the Positive Control**—Dilute TaqPath™ COVID-19 Control (1×10^4 copies/µL) to a working stock of 25 copies/µL.
   a. Pipet 98 µL of TaqPath™ COVID-19 Control Dilution Buffer into a microcentrifuge tube, then add 2 µL of TaqPath™ COVID-19 Control. Mix well, then centrifuge briefly.
   b. Pipet 87.5 µL of TaqPath™ COVID-19 Control Dilution Buffer into a second microcentrifuge tube, then add 12.5 µL of the dilution created in substep 3a. Mix well, then centrifuge briefly.

   Note: The TaqPath™ COVID-19 Control does not contain the MS2 template.
4. **Prepare the Reaction Mix**—For each run, combine the following components sufficient for the number of tests, plus one Positive Control and one Negative Control.

   All volumes include 10% overage for pipette error.

Component	Volume per Sample or Control	Volume for *n* Samples plus 2 Controls	Volume for 94 Samples plus 2 Controls
TaqPath™ 1-Step Multiplex Master Mix (No ROX™) (4X)	6.25 µL	6.875 x (*n* + 2) µL	660 µL
COVID-19 Real Time PCR Assay Multiplex	1.25 µL	1.375 x (*n* + 2) µL	132 µL
Nuclease-free Water	12.50 µL	13.75 x (*n* + 2) µL	1320 µL
Total Reaction Mix volume	**20.0 µL**	**—**	**2112 µL**

5. **Set up the reaction plate**—Pipette 20.0 µL of the Reaction Mix prepared in step 4 into each well of a MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL, then combine with the Sample or the Control according to the following table.

Component	Volume per reaction		
	Sample reaction	Purified Positive Control (TaqPath™ COVID-19 Control)	Negative Control reaction
Reaction Mix	20.0 µL	20.0 µL	20.0 µL
Purified Sample nucleic acid	5.0 µL	—	—
Positive Control (TaqPath™ COVID-19 Control)[1]	—	2.0 µL	—
Nuclease-free Water	—	3.0 µL	—
Purified Negative Control	—	—	5.0 µL
Total volume	**25.0 µL**	**25.0 µL**	**25.0 µL**

[1] From step 3.

Set up and run the Applied Biosystems™ 7500 Fast Dx Real-Time PCR instrument

See the *Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument Reference Guide* (Pub. No. 4406991) for detailed instructions.

The instrument must be calibrated for ABY™ dye and JUN™ dye.

1. From the laptop or tower computer, open the SDT file.

   The SDT file is at the following location: `<installation directory>\Applied Biosystems\COVID-19 Interpretive Software Client\docs\User Documents`.

   The SDT file contains the settings for the run.

2. Confirm the run settings.
   - Assay: Standard Curve (Absolute Quantitation)
   - Run mode: Standard 7500
   - Passive reference: None
   - Sample volume: 25 µL

   IMPORTANT! The passive reference must be set to **None**.

3. Using the **Detector Manager** in the **Tools** menu create the following detectors with the quencher set as none. The detector name must be an exact match with the names shown in the table below, including upper and lower case letters.

Reporter dye	Detector
FAM	ORF1ab
VIC	N gene
ABY	S gene
JUN	MS2

4. Set up the plate layout by assigning a unique sample name to each well.

5. Assign a **Task** to each well.
   - **Unknown**—for patient samples
   - **Standard**—for Positive Control
   - **NTC**—for Negative Control

   IMPORTANT! Ensure that **Standard** is used for the Positive Control and that **NTC** is used for the Negative Control.

6. Confirm the thermal protocol.

Step	Temperature	Time	Number of cycles
UNG incubation	25°C	2 minutes	1
Reverse transcription	53°C	10 minutes	1
Activation	95°C	2 minutes	1
Denaturation	95°C	3 seconds	40
Anneal / extension	60°C	30 seconds	

7. Click **Save As**, enter a file name, then click **Save**.

8. In the **Reason for Change Entry** dialog box, enter a description, then click **OK**.

9. Reopen the file, load the plate, then start the run on the Applied Biosystems™ 7500 Fast Dx Real-Time PCR instrument.

Analyze the data

(*Required*) To obtain the Applied Biosystems™ COVID-19 Interpretive Software, contact **InstrumentServices@thermofisher.com** or 1 800 955 6288 (Select option 3, then option 1).

For detailed instructions about using the software, click the **Help** menu to access the *COVID-19 Interpretive Software Help*.

1. In the COVID-19 Interpretive Software **Home** screen, click the **Import Samples** button.
2. Select the SDS files to import, then click **Open**.
   After import, the software analyzes the run data, performs Quality Check (QC) analysis, and calculates the interpretive results for each sample and control.
3. In the **Batches** pane of the **Home** screen, select a batch to view the status and result for each sample in the **Samples** list.
4. To generate a batch export file (CSV or XLSX), select the checkbox for the batch, then click the **Export Batch** button at the top of the **Home** screen. Click **Open folder location** in the dialog box, then navigate to the exported file.
5. To generate a batch report file (PDF), select the checkbox for the batch, then click the **Report Batch** button at the top of the **Home** screen. Click **Open folder location** in the dialog box, then navigate to the report.

Results interpretation for patient samples

Results interpretation is performed by the Applied Biosystems™ COVID-19 Interpretive Software.

Table 3 Result interpretation for patient samples

ORF1ab	N gene	S gene	MS2	Status	Result	Action
NEG	NEG	NEG	NEG	Invalid	NA	Repeat test. If the repeat result remains invalid, consider collecting a new specimen.
NEG	NEG	NEG	POS	Valid	SARS-CoV-2 Not Detected	Report results to healthcare provider. Consider testing for other viruses
Only one SARS-CoV-2 target = POS			POS or NEG	Valid	SARS-CoV-2 Inconclusive[1]	Repeat test. If the repeat result remains inconclusive, contact CDC immediately for instructions for transfer of the specimen to CDC for additional testing and guidance.
Two or more SARS-CoV-2 targets = POS			POS or NEG	Valid	Presumptive Positive SARS-CoV-2	Report results to healthcare provider and CDC. Contact CDC immediately for instructions for transfer of the specimen to CDC for additional testing and guidance.

[1] Samples with a result of SARS-CoV-2 Inconclusive shall be retested one time.

Assay limitations

- The use of this assay as an *In vitro* diagnostic under the FDA Emergency Use Authorization (EUA) is limited to laboratories that are certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. § 263a, to perform high complexity tests.
- The TaqPath™ RT-PCR COVID-19 Kit performance was established using nasopharyngeal swab, nasopharyngeal aspirate, and bronchoalveolar lavage samples only. Other specimen types have not been evaluated and should not be tested with this assay.
- Samples must be collected, transported, and stored using appropriate procedures and conditions. Improper collection, transport, or storage of specimens may hinder the ability of the assay to detect the target sequences.
- Extraction and amplification of nucleic acid from clinical samples must be performed according the specified methods listed in this procedure. Other extraction approaches and processing systems have not been evaluated.
- False-negative results may arise from:
  - Improper sample collection
  - Degradation of the viral RNA during shipping/storage
  - Specimen collection after nucleic acid can no longer be found in the specimen matrix
  - Using unauthorized extraction or assay reagents
  - The presence of RT-PCR inhibitors
  - Mutation in the SARS-CoV-2 virus

  - Failure to follow instructions for use
- False-positive results may arise from:
  - Cross contamination during specimen handling or preparation
  - Cross contamination between patient samples
  - Specimen mix-up
  - RNA contamination during product handling
- The impacts of vaccines, antiviral therapeutics, antibiotics, chemotherapeutic or immunosuppressant drugs have not been evaluated. The TaqPath™ RT-PCR COVID-19 Kit cannot rule out diseases caused by other bacterial or viral pathogens.
- Negative results do not preclude infection with SARS-CoV-2 virus, and should not be the sole basis of a patient management decision.
- A patient matched serum specimen is required for serological follow up testing of all positive RT-PCR results, per the CDC testing algorithm.
- Laboratories are required to report all positive results to the appropriate public health authorities.

Conditions of authorization for labs

The TaqPath™ RT-PCR COVID-19 Kit Letter of Authorization, along with the authorized Fact Sheet for Healthcare Providers, the authorized Fact Sheet for Patients, and authorized labeling are available on the FDA website: **https://www.fda.gov/MedicalDevices/Safety/EmergencySituations/ucm161496.htm**.

However, to assist clinical laboratories using the TaqPath™ RT-PCR COVID-19 Kit, the relevant Conditions of Authorization are listed below.

- Authorized laboratories will include with reports of the results of the TaqPath™ RT-PCR COVID-19 Kit the authorized Fact Sheet for Healthcare Providers and the authorized Fact Sheet for Patients. Under exigent circumstances, other appropriate methods for disseminating these Fact Sheets may be used, which may include mass media.
- Authorized laboratories will perform RT-PCR with the TaqPath™ RT-PCR COVID-19 Kit using sample collected via nasopharyngeal swab, nasopharyngeal aspirate, bronchoalveolar lavage, or other authorized specimen types.
- Authorized laboratories will perform RT-PCR with the TaqPath™ RT-PCR COVID-19 Kit using samples extracted using the KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head and MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit or with other authorized extraction methods.
- Authorized laboratories will perform RT-PCR with the TaqPath™ RT-PCR COVID-19 Kit on the Applied Biosystems™ 7500 Fast Dx Real-Time PCR instrument, or other authorized instruments.
- Authorized laboratories will have a process in place for reporting test results to healthcare providers and relevant public health authorities, as appropriate.
- Authorized laboratories will collect information on the performance of the test and report to DMD/OIR/CDRH (via email **CDRH-EUA-Reporting@fda.hhs.gov**) and Thermo Fisher Scientific any suspected occurrence of false positive or false negative results of which they become aware.
- All laboratory personnel using the test should be appropriately trained in RT-PCR techniques and use appropriate laboratory and personal protective equipment when handling this kit, and use the test in accordance with the authorized labeling.
- Thermo Fisher Scientific, its authorized distributor(s), and authorized laboratories, will ensure that any records associated with this EUA are maintained until notified by FDA. Such records will be made available to FDA for inspection upon request.

Performance characteristics

Analytical performance of the TaqPath™ RT-PCR COVID-19 Kit was evaluated by determining limit of detection (LoD), characterizing the impact of interfering substances and cross-reactivity, as described in the following sections.

Limit of detection (LoD)

The LoD study established the lowest SARS-CoV-2 viral concentration (Genomic Copy Equivalents or GCE) that can be detected by the TaqPath™ COVID-19 Combo Kit in a particular specimen type at least 95% of the time. Banked Nasopharyngeal swab (NP) and Bronchoalveolar lavage (BAL) samples, obtained from U.S. patients in the years 2015-2019, were pooled, respectively, and spiked with purified SARS-CoV-2 RNA at several concentrations and processed through the TaqPath™ COVID-19 Combo Kit workflow. A three-phase approach was used to determine the LoD for each specimen type.

Table 4 LoD results

Specimen type	Limit of Detection (GCE/mL)	Limit of Detection (GCE/reaction)
Bronchoalveolar lavage	250 GCE/mL	10 GCE/reaction
Nasopharyngeal swab	250 GCE/mL	10 GCE/reaction

Reactivity (Inclusivity)

The assays were mapped to 185 complete SARS-CoV-2 genomes of human host in GenBank and GISAID databases as of March 5, 2020. Primer and probes sequences for SARS-CoV-2 ORF1ab, S gene, and N gene assays had 100% homology to all SARS-CoV-2 isolates analyzed, with one exception. EPI_ISL_407084 showed a mismatch at position 7 from the 5' end of the reverse primer (23 nt length) corresponding to 95.6% homology. The mismatch is located at the 5'end of the primer and does not affect the test performance.

Cross-reactivity

In silico analysis of the following forty-two (42) organisms:

Table 5 Organisms used for *in silico* cross-reactivity analysis

Organism
Human coronavirus 229E
Human coronavirus OC43
Human coronavirus HKU1
Human coronavirus NL63
SARS-coronavirus
MERS-coronavirus
Adenovirus
Human Metapneumovirus (hMPV)
Parainfluenza 1
Parainfluenza 2
Parainfluenza 3
Parainfluenza 4
Influenza A
Influenza B
Influenza C
Enterovirus
Respiratory Syncytial Virus A
Respiratory Syncytial Virus B
Rhinovirus/Enterovirus
Parechovirus
Candida albicans
Corynebacterium diphtheriae
Legionella (non-pneumophila)
Bacillus anthracis (Anthrax)
Moraxella catarrhalis
Neisseria elongata and *N. meningitidis*
Pseudomonas aeruginosa
Staphylococcus epidermidis
Streptococcus salivarius
Leptospira sp.
Chlamydophila pneumoniae
Chlamydophila psittaci
Coxiella burnetii (Q-Fever)
Staphylococcus aureus
Haemophilus influenzae
Legionella pneumophila

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Organism
Mycobacterium tuberculosis
Streptococcus pneumoniae
Streptococcus pyogenes
Bordetella pertussis
Mycoplasma pneumoniae
Pneumocystis jirovecii (PJP)

Blast analysis showed ≥80% homology for one assay component (forward primer, reverse primer, or probe) for select isolates. Despite ≥80% homology of one assay component for select isolates, there is no anticipated amplification because hybridization of all three assay components are necessary to generate a signal. We also found instances where different assay components had ≥80% homology to different isolates of the same species. For example, *Bacillus anthracis* strain AFS029987 had ≥80% homology to the ORF1ab forward primer while strain MCCC 1A01412 had ≥80% homology to the ORF1ab reverse primer. Since these are two different organisms, amplification will not occur. The *in silico* analysis indicates that significant amplification of non-target sequences will not occur that result in cross-reactivity or potentially interfere with detection of SARS-CoV-2.

Clinical evaluation

A clinical evaluation study was performed to evaluate the performance of the TaqPath™ RT-PCR COVID-19 Kit using nasopharyngeal swab (NP) and bronchoalveolar lavage (BAL) specimens.

A total of sixty (60) contrived positive specimens were tested:

- 30 contrived positive nasopharyngeal swab (NP) specimens
- 30 contrived positive bronchoalveolar lavage (BAL) specimens

Samples were contrived by spiking known concentrations of isolated SARS-CoV-2 RNA, relative to the product LoD, into matrices which were determined to be negative by the TaqPath™ RT-PCR COVID-19 Kit prior to spiking in the RNA.

In addition to the contrived positive specimens, sixty (60) negative specimens were tested:

- 30 negative nasopharyngeal swab (NP) specimens
- 30 negative samples bronchoalveolar lavage (BAL) specimens

Final clinical evaluation study outcome as shown below:

Table 6 BAL Clinical Evaluation Study

Final RNA Concentration in Sample	Interpretation
5X LoD	5/5 presumptive positive
3X LoD	5/5 presumptive positive
2X LoD	20/20 presumptive positive
Negative	30/30 not detected

Table 7 NP Clinical Evaluation Study

Final RNA Concentration in Sample	Interpretation
5X LoD	5/5 presumptive positive
3X LoD	5/5 presumptive positive
2X LoD	20/20 presumptive positive
Negative	30/30 not detected

Related documentation

Document	Publication Number
Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument Reference Guide	4406991
MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (automated extraction) User Guide	MAN0018073
COVID-19 Interpretive Software Installation Quick Reference	MAN0019184
TaqPath™ COVID-19 Combo Kit Instructions for Use	100092995

Customer and technical support

Visit **thermofisher.com/support** for the latest service and support information.

- Worldwide contact telephone numbers
- Product support information
  - Product FAQs
  - Software, patches, and updates
  - Training for many applications and instruments
- Order and web support
- Product documentation
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

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Revision history: Pub. No. MAN0019181

Revision	Date	Description
A.0	12 March 2020	New document.

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APPENDIX 14

appliedbiosystems

TaqPath™ COVID-19 Combo Kit and TaqPath™ COVID-19 Combo Kit Advanced*

INSTRUCTIONS FOR USE

Multiplex real-time RT-PCR test intended for the qualitative detection of nucleic acid from SARS-CoV-2

*TaqPath™ COVID-19 Combo Kit Advanced reagent volumes have been optimized for workflows that use 14.0 µL or 17.5 µL of purified sample RNA

Catalog Numbers A47813 and A47814
Publication Number MAN0019181
Revision H.01

IVD

For In Vitro Diagnostic Use. For Emergency Use Authorization Only | Rx Only

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Revision history: Pub. No. MAN0019181

Revision	Date	Description
H.01	10 December 2020	Internal document control update only, with no changes to Instructions for Use rev. H.0 released on DCO1199965 and posted to the thermofisher.com website. Update to align the 20 Nov 2020 FDA-released Instructions for Use with removal of the licensing statement covering Limited Use Label Licenses on page 2.
H.0	10 November 2020	• The instructions for using the TaqPath™ COVID-19 Combo Kit Advanced were updated to indicate that the kit is now compatible with all instruments. • The compatible COVID-19 Interpretive Software versions were updated to v1.5 and 2.5, which include amplification curves. • Instructions were added on what to do in the event of bead carry over from RNA extraction. • The licensing statement covering Limited Use Label Licenses was removed from page 2.
G.0	9 October 2020	• Guidance and protocols were added for samples collected using the Everlywell™ COVID-19 Test Home Collection Kit, including use of the new TaqMan™ SARS-CoV-2 RNase P Assay Kit. • Information and protocols were added for the TaqPath™ COVID-19 Combo Kit Advanced (Cat. No. A47813), which supports preparation of RT-PCR reactions from 17.5 µL and 14.0 µL of purified sample RNA. • "Intended Use" on page 9 and Chapter 12, "Conditions of authorization for labs" were updated with information for the Everlywell™ COVID-19 Test Home Collection Kit and the TaqPath™ COVID-19 Combo Kit Advanced. • COVID-19 Interpretive Software v1.4 and v.2.4 were added to support use of the RNase P assay, and instructions for obtaining the software were updated. • Minor corrections were made to the kit configuration information for the TaqPath™ COVID-19 Combo Kit. • General laboratory recommendations were added (see page 17). • The name of the Wash Buffer was changed to Wash Solution in the RNA extraction procedures. A note was added that the Wash Solution can develop particulates but this does not affect performance. • "Interpretation of the results" on page 104 was updated to include results for the RNase P assay and guidance on reporting results to the appropriate public health authorities. • Minor updates were made to the Intended Use.
F.0	15 July 2020	• Added Applied Biosystems™ COVID-19 Interpretive Software v1.3 and Applied Biosystems™ COVID-19 Interpretive Software v2.3. • Removed Applied Biosystems™ COVID-19 Interpretive Software v1.2, Applied Biosystems™ COVID-19 Interpretive Software v2.0, and Applied Biosystems™ COVID-19 Interpretive Software v2.2. • Added C_t cutoff information (Appendix B, "Ct cutoff values for assay targets"). • Removed instructions to mix by pipetting up and down 10 times when preparing RT-PCR plates. Added instructions to vortex the plates to ensure proper mixing. • Updated centrifuge and sealing instructions when preparing the RT-PCR plates. • Updated instructions to create a unique name for each well in the physical plate, not just the wells with a patient sample. • Updated reactivity (inclusivity) (page 113) and the warnings and precautions (page 15). • Specified that retesting must be performed by re-extracting the original sample and repeating the RT-PCR ("Interpretation of the results" on page 104).

Revision	Date	Description
E.0	12 May 2020	Added the following products as an alternative to the KingFisher™ 96 KF microplate for the tip comb plate: • Tip Comb Presenting Plate for KF 96 • Nunc™ MicroWell™ 96-Well Microplate, barcoded • Nunc™ MicroWell™ 96-Well Microplate, Flat Bottom • Nunc™ F96 MicroWell™ Black Polystyrene Plate • KingFisher™ Deep-Well 96 Plate
D.0	11 May 2020	• Updated the list of acceptable sample types and added specimen storage conditions. • Removed catalog numbers for combo kit components. • Updated materials listed in "Required materials not supplied" on page 11. • Updated the automated RNA extraction procedure with new plastics, new instrument programs, and revised wash and plate handling steps. • Updated the manual RNA extraction procedure with an optional ethanol volume for 200-µL sample input volumes, and an option to use the MagMAX™ Viral/Pathogen II Nucleic Acid Isolation Kit for 400-µL sample input volumes. • Updated the real-time RT-PCR preparation procedure with new instructions for 384-well plates, clarified guidelines for negative controls, and optional plates without barcodes. • Added procedures for the QuantStudio™ 7 Flex Real-Time PCR Instrument (384-well block) and the QuantStudio™ 5 Real-Time PCR Instrument (384-well block). • Added instrument firmware requirements and removed calibration plates for the QuantStudio™ 5 Real-Time PCR Instrument. • Added Applied Biosystems™ COVID-19 Interpretive Software v2.2. • Updated control requirements in interpretation of results, based on addition of 384-well plates.
C.0	20 April 2020	• Removed 100-reaction kit. • Added a catalog number for the KingFisher™ Deep-Well 96 Plate. • Updated the catalog number for the Compact Digital Microplate Shaker. • Added catalog number for the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit and removed catalog numbers for individual components of the kit. • Added the MagMAX™ Viral/Pathogen II Nucleic Acid Isolation Kit. • Added an option to extract RNA with 200 µL of sample. • Added Applied Biosystems™ 7500 Real-Time PCR Instrument and Applied Biosystems™ QuantStudio™ 5 Real-Time PCR Instruments. • Added Applied Biosystems™ COVID-19 Interpretive Software v1.2 and Applied Biosystems™ COVID-19 Interpretive Software v2.0. • Removed Applied Biosystems™ COVID-19 Interpretive Software v1.0 and Applied Biosystems™ and COVID-19 Interpretive Software v1.1. • Added specific instructions to vortex and centrifuge the reaction plate for RT-PCR ("Prepare the RT-PCR reactions (400-µL sample input, 96-well reaction plate, COVID-19 assay only)" on page 39 and "Prepare the RT-PCR reactions (200-µL sample input, 96-well reaction plate, COVID-19 assay only)" on page 35). • Specified that retesting must be done with the original sample ("Interpretation of the results" on page 104). • Reorganized the content to perform RT-PCR based on the real-time PCR instrument. • Added "Interfering substances" on page 113. • Added information to customer and technical support (page 122).

Revision	Date	Description
B.0	24 March 2020	• Added MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit components for 1,000 preparations to Required Materials. • Added manual RNA extraction protocol and required materials for the manual RNA extraction protocol. • Added Applied Biosystems™ 7500 Fast Real-Time PCR Instrument. • Removed storage options for RNA after extraction. • Updated guidelines for RT-PCR to run the plate immediately after preparation and to keep the plate on ice until it is loaded into the real-time PCR instrument. • When setting up the RT-PCR reaction, added instructions to mix by pipetting up and down 10 times and seal and centrifuge the reaction plate. • Added COVID-19 Interpretive Software v1.1 (compatible with Applied Biosystems™ 7500 Fast Real-Time PCR Instrument and Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument). • Added that the run file must be opened, analyzed, and saved in the instrument software before it is opened in COVID-19 Interpretive Software. • For TaqPath™ COVID-19 RT-PCR Kit, 1,000 reactions (Cat. No. A47817), changed MS2 Phage Control from 20 tubes × 500 µL to 10 tubes × 1,000 µL. • Updated instructions to obtain the COVID-19 Interpretive Software. • Changed Limit of Detection and Clinical Evaluation data to 1 decimal place.
A.0	15 March 2020	New document.

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TaqPath™ COVID-19 Combo Kit and TaqPath™ COVID-19 Combo Kit Advanced product information

Intended Use

The TaqPath™ COVID-19 Combo Kit, which can be labeled as the TaqPath™ COVID-19 Combo Kit Advanced, contains the assays and controls for a real-time reverse transcription polymerase chain reaction (RT-PCR) test intended for the qualitative detection of nucleic acid from SARS-CoV-2 in upper respiratory specimens (such as nasopharyngeal, oropharyngeal, nasal, and mid-turbinate swabs, and nasopharyngeal aspirate) and bronchoalveolar lavage (BAL) specimens from individuals suspected of COVID-19 by their healthcare provider. Testing is limited to laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, that meet requirements to perform high complexity tests.

This test is also authorized for use with the Everlywell™ COVID-19 Test Home Collection Kit for individuals to self-collect nasal swab specimens when determined by a healthcare provider to be appropriate based on results of a COVID-19 questionnaire.

Results are for the identification of SARS-CoV-2 RNA. The SARS-CoV-2 RNA is generally detectable in upper respiratory and bronchoalveolar lavage (BAL) specimens during the acute phase of infection. Positive results are indicative of the presence of SARS-CoV-2 RNA; clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status. Positive results do not rule out bacterial infection or co-infection with other viruses. The agent detected may not be the definite cause of disease. Laboratories within the United States and its territories are required to report all test results to the appropriate public health authorities.

Negative results do not preclude SARS-CoV-2 infection and should not be used as the sole basis for patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

Testing with the TaqPath™ COVID-19 Combo Kit and the TaqPath™ COVID-19 Combo Kit Advanced are intended for use by qualified and trained clinical laboratory personnel specifically instructed and trained in the techniques of real-time PCR and *in vitro* diagnostic procedures. The TaqPath™ COVID-19 Combo Kit and the TaqPath™ COVID-19 Combo Kit Advanced are only for use under the Food and Drug Administration's Emergency Use Authorization.

Product description

The TaqPath™ COVID-19 Combo Kit and TaqPath™ COVID-19 Combo Kit Advanced include the assays and controls for a multiplex real-time RT-PCR test for the qualitative detection of RNA from SARS-CoV-2 in upper respiratory specimens (such as nasopharyngeal, oropharyngeal, nasal, and mid-turbinate swabs, and nasopharyngeal aspirate) and bronchoalveolar lavage (BAL) specimens from individuals suspected of COVID-19 by their healthcare provider.

Each kit includes the following components:

- Multiplexed assays that contain three primer/probe sets specific to different SARS-CoV-2 genomic regions and primers/probes for bacteriophage MS2
- MS2 Phage Control as an internal process control for nucleic acid extraction
- TaqPath™ COVID-19 Control as a positive RNA control that contains targets specific to the SARS-CoV-2 genomic regions targeted by the assays

Contents and storage

Table 1 TaqPath™ COVID-19 Combo Kit, 1,000 reactions (Cat. No. A47814)

Box	Components	Amount	Storage
TaqPath™ COVID-19 RT-PCR Kit	COVID-19 Real Time PCR Assay Multiplex (ORF1ab, N gene, S gene, MS2)	1,500 µL	–30°C to –10°C
	MS2 Phage Control	10 × 1,000 µL	–30°C to –10°C
TaqPath™ COVID-19 Control (1 x 10^4 copies/µL)		2 × 10 µL per box; 5 boxes per kit	≤ –70°C
TaqPath™ COVID-19 Control Dilution Buffer		2 × 250 µL per box; 5 boxes per kit	–30°C to –10°C

Table 2 TaqPath™ COVID-19 Combo Kit Advanced, 200 reactions (Cat. No. A47813)

Box	Components	Amount	Storage
TaqPath™ COVID-19 RT-PCR Kit Advanced	COVID-19 Assay Multiplex Advanced (ORF1ab, N gene, S gene, MS2)	300 µL	–30°C to –10°C
	MS2 Phage Control	2 × 1,000 µL	–30°C to –10°C
TaqPath™ COVID-19 Control (1 x 10^4 copies/µL)		2 × 10 µL	≤ –70°C
TaqPath™ COVID-19 Control Dilution Buffer		2 × 250 µL	–30°C to –10°C

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Catalog numbers that appear as links open the web pages for those products.

Item	Source
Real-time PCR instrument	
Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument (used with SDS Software v1.4.1)	4406984 (with laptop computer) 4406985 (with tower computer)
Applied Biosystems™ 7500 Fast Real-Time PCR Instrument (used with SDS Software v1.5.1 or 7500 Software v2.3)	4351106 (with laptop computer) 4351107 (with desktop computer)
Applied Biosystems™ 7500 Real-Time PCR Instrument (used with 7500 Software v2.3)	4351104 (with laptop computer) 4351105 (with desktop computer)
Applied Biosystems™ QuantStudio™ 5 Real-Time PCR Instrument, 96-well, 0.2-mL block (used with QuantStudio™ Design and Analysis Desktop Software v1.5.1)	A28569 (with laptop computer) A28574 (with desktop computer) A28139 (instrument only)
Applied Biosystems™ QuantStudio™ 5 Real-Time PCR Instrument, 96-well, 0.1-mL block (used with QuantStudio™ Design and Analysis Desktop Software v1.5.1)	A28568 (with laptop computer) A28573 (with desktop computer) A28138 (instrument only)
Applied Biosystems™ QuantStudio™ 5 Real-Time PCR Instrument, 384-well block (used with QuantStudio™ Design and Analysis Desktop Software v1.5.1)	A28570 (with laptop computer) A28575 (with desktop computer) A28140 (instrument only)
Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR Instrument, 384–well block (used with QuantStudio™ Real-Time PCR Software v1.3)	4485695 (with laptop computer) 4485701 (with desktop computer)
Equipment	
Laboratory freezers • –30°C to –10°C • ≤ –70°C	MLS
Centrifuge, with a rotor that accommodates standard and deepwell microplates	MLS
Microcentrifuge	MLS
Laboratory mixer, vortex or equivalent	MLS

(continued)

Item	Source
Single and multichannel adjustable pipettors (1.00 µL to 1,000.0 µL)	MLS
Cold block (96-well or 384-well) or ice	MLS
Automated nucleic acid extraction system and materials	
KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head	5400630
KingFisher™ Flex 96 Deep-Well Heating Block	24075430
KingFisher™ Deep-Well 96 Plate	95040450, A48305, A48424, 95040455
96-well plate for the tip comb, one of the following: • KingFisher™ 96 KF microplate • Tip Comb Presenting Plate for KF 96 • Nunc™ MicroWell™ 96-Well Microplate, Flat Bottom • Nunc™ MicroWell™ 96-Well Microplate, barcoded • ABgene™ 96–Well Polypropylene Storage Microplate • ABgene™ 96–Well 1.2–mL Polypropylene Deepwell Storage Plate • Nunc™ F96 MicroWell™ Black Polystyrene Plate • Nunc™ F96 MicroWell™ White Polystyrene Plate • KingFisher™ Deep-Well 96 Plate	 • 97002540 • 267600 • 167008 • 269787 • AB0796 • AB1127 • 137101 • 136101 • 95040450, A48305, A48424, 95040455
KingFisher™ 96 tip comb for DW magnets	97002534, A48438, A48414
Manual nucleic acid extraction system and materials	
Magnetic Stand-96	AM10027 AM10050
Compact Digital Microplate Shaker	88882005
Incubator capable of reaching 65°C with slatted shelves	MLS
KingFisher™ Deep-Well 96 Plate	95040450, A48305, A48424, 95040455
Standard 96-well plate for the eluate, one of the following: • KingFisher™ 96 KF microplate • MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL • MicroAmp™ Fast Optical 96-Well Reaction Plate, 0.1 mL • MicroAmp™ Optical 96-Well Reaction Plate with Barcode, 0.2 mL • MicroAmp™ Optical 96-Well Reaction Plate, 0.2 mL	 • 97002540 • 4346906, 4366932 • 4346907 • 4306737, 4326659 • N8010560, 4316813

(continued)

Item	Source
MicroAmp™ Clear Adhesive Film	4306311
Kits and reagents	
Required if specimens were collected using the Everlywell™ COVID-19 Test Home Collection Kit: TaqMan™ SARS-CoV-2 RNase P Assay Kit	A49564
MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (up to 200 preparations, when 200 µL of sample is used)	A42352
MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (up to 2,000 preparations, when 200 µL of sample is used)	A48310
MagMAX™ Viral/Pathogen II Nucleic Acid Isolation Kit (up to 2,000 preparations, when 200 µL of sample is used)	A48383
TaqPath™ 1-Step Multiplex Master Mix (No ROX™)	A28521, A28522, A28523
Fisher BioReagents™ Ethanol, Absolute, Molecular Biology Grade[1], or equivalent	BP2818100, BP2818500, BP28184
Nuclease-free Water (not DEPC-Treated)	MLS
Calibration plates (7500 Real-Time PCR Instrument series)	
ABY™ Dye Spectral Calibration Plate for Multiplex qPCR, Fast 96-well (0.1-mL)	A24734
JUN™ Dye Spectral Calibration Plate for Multiplex qPCR, Fast 96-well (0.1-mL)	A24735
ABY™ Dye Spectral Calibration Plate for Multiplex qPCR, 96-well (0.2-mL)	A24738
JUN™ Dye Spectral Calibration Plate for Multiplex qPCR, 96-well (0.2-mL)	A24737
Calibration plates (QuantStudio™ 7 Flex Real-Time PCR Instrument)	
ABY™ Dye Spectral Calibration Plate for Multiplex qPCR, 384-well	A24736
JUN™ Dye Spectral Calibration Plate for Multiplex qPCR, 384-well	A24733
Tubes, plates, and other consumables	
MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL	4346906, 4366932
MicroAmp™ Fast Optical 96-Well Reaction Plate, 0.1 mL	4346907

(continued)

Item	Source
MicroAmp™ Optical 96-Well Reaction Plate with Barcode, 0.2 mL	4306737, 4326659
MicroAmp™ Optical 96-Well Reaction Plate, 0.2 mL	N8010560, 4316813
MicroAmp™ Optical 384-Well Reaction Plate with Barcode	4309849, 4326270, 4343814
MicroAmp™ Optical 384-Well Reaction Plate	4343370
MicroAmp™ Clear Adhesive Film	4306311
MicroAmp™ Optical Adhesive Film	4311971, 4360954
MicroAmp™ Adhesive Film Applicator	4333183
Nonstick, RNase-free microcentrifuge tubes (1.5 mL and 2.0 mL)	**thermofisher.com/plastics**
Sterile aerosol barrier (filtered) pipette tips	**thermofisher.com/pipettetips**

[1] Available at **fisherscientific.com**.

Instrument, assay, and software compatibility

The following table lists the version of the Applied Biosystems™ COVID-19 Interpretive Software that is compatible with your instrument, its associated analysis software, and whether the TaqMan™ SARS-CoV-2 RNase P Assay is used in the test procedure.

Note: The TaqMan™ SARS-CoV-2 RNase P Assay is required for specimens collected using the Everlywell™ COVID-19 Test Home Collection Kit.

For information on how to obtain the Applied Biosystems™ COVID-19 Interpretive Software, see "Obtain the Applied Biosystems™ COVID-19 Interpretive Software" on page 103.

To obtain the analysis software or firmware for your real-time PCR instrument, go to **thermofisher.com/qpcrsoftware**, then select your instrument in the **Real-Time PCR** section.

Instrument	Analysis software used with the instrument	Minimum compatible COVID-19 Interpretive Software version
7500 Fast Dx Real-Time PCR Instrument	SDS Software v1.4.1	v1.5
7500 Fast Real-Time PCR Instrument	SDS Software v1.5.1 *or* 7500 Software v2.3	v1.5
7500 Real-Time PCR Instrument	7500 Software v2.3	v1.5

(continued)

Instrument	Analysis software used with the instrument	Minimum compatible COVID-19 Interpretive Software version
QuantStudio™ 5 Real-Time PCR Instrument with instrument firmware v1.3.3 • 96-well, 0.2-mL block • 96-well, 0.1-mL block • 384-well block	QuantStudio™ Design and Analysis Desktop Software v1.5.1	v2.5
QuantStudio™ 7 Flex Real-Time PCR Instrument with instrument firmware v1.0.4 • 384-well block	QuantStudio™ Real-Time PCR Software v1.3	v2.5

Warnings and precautions

The TaqPath™ COVID-19 RT-PCR Kit and the TaqPath™ COVID-19 RT-PCR Kit Advanced workflows should be performed by qualified and trained staff to avoid the risk of erroneous results. Use separate areas for the preparation of patient samples and controls to prevent false positive results. Samples and reagents must be handled in a biological safety cabinet.

- The assay is for *in vitro* diagnostic use under the FDA Emergency Use Authorization Only.
- This test has not been FDA cleared or approved.
- This test has been authorized only for the detection of nucleic acid from SARS-CoV-2, not for any other viruses or pathogens.
- This test is only authorized for the duration of the declaration that circumstances exist justifying the authorization of emergency use of *in vitro* diagnostics for detection and/or diagnosis of COVID-19 under Section 564(b)(1) of the Act, 21 U.S.C. § 360bbb-3(b)(1), unless the authorization is terminated or revoked sooner.
- Samples and controls should always be treated as if infectious and/or biohazardous in accordance with safe laboratory procedures.
- Follow necessary precautions when handling specimens. Use personal protective equipment (PPE) consistent with current guidelines for the handling of potentially infectious samples.
- Always use pipette tips with aerosol barriers. Tips that are used must be sterile and free from DNases and RNases.
- Do not eat, drink, smoke, or apply cosmetic products in the work areas.
- Modifications to assay reagents, assay protocol, or instrumentation are not permitted, and are in violation of the product Emergency Use Authorization.
- Reagents must be stored and handled as specified in “Contents and storage” on page 10.
- Do not use the kits after the indicated expiry date.
- Dispose of waste in compliance with local, state, and federal regulations.
- Safety Data Sheets are available upon request.

- Laboratories within the United States and its territories are required to report all test results to the appropriate public health authorities.
- Positive results are indicative of the presence of SARS-CoV-2 RNA.

Assay limitations

- The use of this assay as an *In vitro* diagnostic under the FDA Emergency Use Authorization (EUA) is limited to laboratories that are certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. § 263a, that meet requirements to perform high complexity tests.
- The TaqPath™ COVID-19 RT-PCR Kit and the TaqPath™ COVID-19 RT-PCR Kit Advanced performance was established using nasopharyngeal and oropharyngeal swab, nasopharyngeal aspirate, and bronchoalveolar lavage samples only. Nasal swabs and mid-turbinate swabs are considered acceptable specimen types for use with the TaqPath™ COVID-19 RT-PCR Kit and the TaqPath™ COVID-19 RT-PCR Kit Advanced, but performance with these specimen types has not been established. Refer to FDA's *FAQs on Diagnostic Testing for SARS-CoV-2* for additional information. Specimen types other than nasopharyngeal, oropharyngeal, nasal and mid-turbinate nasal swabs, nasopharyngeal aspirate, and bronchoalveolar lavage should not be tested with this assay.
- Samples must be collected, transported, and stored using appropriate procedures and conditions. Improper collection, transport, or storage of specimens may hinder the ability of the assay to detect the target sequences.
- Extraction and amplification of nucleic acid from clinical samples must be performed according the specified methods listed in this procedure. Other extraction approaches and processing systems have not been evaluated.
- Specimens submitted using the Everlywell™ COVID-19 Test Home Collection Kit must be tested using the TaqMan™ SARS-CoV-2 RNase P Assay Kit and the TaqPath™ COVID-19 RT-PCR Kit.
- False-negative results may arise from:
  - Improper sample collection
  - Degradation of the SARS-CoV-2 RNA during shipping/storage
  - Specimen collection after SARS-CoV-2 RNA can no longer be found in the specimen matrix
  - Using unauthorized extraction or assay reagents
  - The presence of RT-PCR inhibitors
  - Mutation in the SARS-CoV-2 virus
  - Failure to follow instructions for use
- False-positive results may arise from:
  - Cross contamination during specimen handling or preparation
  - Cross contamination between patient samples
  - Specimen mix-up
  - RNA contamination during product handling
- The impacts of vaccines, antiviral therapeutics, antibiotics, chemotherapeutic or immunosuppressant drugs have not been evaluated. The TaqPath™ COVID-19 RT-PCR Kit and the TaqPath™ COVID-19 RT-PCR Kit Advanced cannot rule out diseases caused by other bacterial or viral pathogens.

- Negative results do not preclude infection with SARS-CoV-2 virus, and should not be the sole basis of a patient management decision.
- Laboratories are required to report all test results to the appropriate public health authorities.
- Detection of RNase P indicates that human nucleic acid is present and implies that human biological material was collected and successfully extracted and amplified. It does not necessarily indicate that the specimen is of appropriate quality to enable detection of SARS-CoV-2.

General laboratory recommendations

- Implement standard operating procedures in your laboratory to prevent contamination, such as the following:
  - Frequent glove changes
  - Frequent decontamination of surfaces, equipment, and pipettes with 10% bleach or decontamination solution, followed by 70% ethanol
  - Use of ultraviolet light during biosafety cabinet decontamination (when available)
- To prevent degradation, keep eluted sample RNA, master mixes, assays, and controls on ice or in cold blocks while in use.
- Limit freeze-thaw cycles.
- Aliquot reagents to prevent stock contamination and reduce the number of freeze-thaw cycles.
- After each run, review the amplification curves in the interpretive software for signs of inadequate vortexing or centrifugation. Contact your Applications Support team for additional information or training on data QC in your instrument software.

Samples and controls

Patient samples must be collected according to appropriate laboratory guidelines. Positive and negative test controls must be included to accurately interpret patient test results.

Store patient samples according to CDC guidelines. See the CDC website: **https://www.cdc.gov/coronavirus/2019-ncov/lab/guidelines-clinical-specimens.html**.

Include the following controls:

Control	Used to monitor	Assays
Positive Control (TaqPath™ COVID-19 Control Kit)	RT-PCR reaction setup and reagent integrity	All three SARS-CoV-2 assays
MS2 Phage Control	RNA extraction	MS2 assay
Negative Control	Cross-contamination during RNA extraction and reaction setup	All three SARS-CoV-2 assays
		MS2 assay
TaqMan™ SARS-CoV-2 RNase P Assay control[1]	Sample adequacy	RNase P assay

[1] Used with the Everlywell™ COVID-19 Test Home Collection Kit workflow.

Sample collection, transport, and storage

Note: Handle all samples and controls as if they are capable of transmitting infectious agents.

Workflow

Extract RNA from patient sample

▼

Perform RT-PCR

▼

Analyze data using the Applied Biosystems™ COVID-19 Interpretive Software

▼

Review run control results

▼

Review results interpretation for patient samples

The workflow begins with nucleic acid extraction from upper respiratory specimens (such as nasopharyngeal, oropharyngeal, nasal, and mid-turbinate swabs, and nasopharyngeal aspirate) and bronchoalveolar lavage (BAL) specimens that arrive in the testing site in transport media. Nucleic acids are isolated and purified from the specimens using the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit or the MagMAX™ Viral/Pathogen II Nucleic Acid Isolation Kit. Nucleic acid isolation can be performed manually or via an automated process using the KingFisher™ Flex Purification System (KingFisher). For more information about using the kit, see "Related documentation" on page 122.

The RT-PCR reactions are prepared. For specimens collected using the Everlywell™ COVID-19 Test Home Collection Kit, the TaqMan™ SARS-CoV-2 RNase P Assay Kit is included as an additional control.

The nucleic acid is reverse transcribed into cDNA and amplified using the TaqPath™ COVID-19 RT-PCR Kit or the TaqPath™ COVID-19 RT-PCR Kit Advanced and one of the following real-time PCR instruments:

- Applied Biosystems™ 7500 Fast Dx Real-Time PCR instrument
- Applied Biosystems™ 7500 Fast Real-Time PCR Instrument
- Applied Biosystems™ 7500 Real-Time PCR Instrument
- Applied Biosystems™ QuantStudio™ 5 Real-Time PCR Instrument, 96-well, 0.2–mL block
- Applied Biosystems™ QuantStudio™ 5 Real-Time PCR Instrument, 96-well, 0.1–mL block
- Applied Biosystems™ QuantStudio™ 5 Real-Time PCR Instrument, 384-well block
- Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR Instrument, 384–well block

In the process, the probes anneal to three (3) specific SARS-CoV-2 target sequences located between three (3) unique forward and reverse primers for the following genes:

- ORF1ab
- N Gene
- S Gene

During the extension phase of the PCR cycle, the 5' nuclease activity of Taq polymerase degrades the probe, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. With each cycle, additional reporter dye molecules are cleaved from their respective probes, increasing the fluorescence intensity. Fluorescence intensity is monitored at each PCR cycle by the real-time PCR instrument.

The data are analyzed, then interpreted by the Applied Biosystems™ COVID-19 Interpretive Software.

The workflow options have been updated to reduce the number of consumables. Previous versions of the workflow in previous revisions of this document are still validated to run the TaqPath™ COVID-19 RT-PCR Kit.

2 Extract RNA (automated method)

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- Extract RNA—Automated method (200-µL sample input volume) 21
- Extract RNA—Automated method (400-µL sample input volume) 24

Automated RNA extraction is performed using the KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head and the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit or MagMAX™ Viral/Pathogen II Nucleic Acid Isolation Kit with a sample input volume of 200 µL or 400 µL.

Before you begin

Note: During the wash steps, the Wash Solution may develop inert white or brown particulates that float in solution. This is not a cause for concern and does not negatively affect performance.

- Determine the number of required reactions based on the number of patient samples to be processed, plus one Negative Control per plate.
- Prepare fresh 80% Ethanol using Ethanol, Absolute, Molecular Biology Grade and Nuclease-free Water (not DEPC-Treated) for the required number of reactions, sufficient for 1 mL per reaction, plus 10% overage.
- Label the short side of each KingFisher™ Deep-Well 96 Plate (4):

Label	Number of plates
Sample plate	1
Wash 1	1
Wash 2	1
Elution plate	1

- Label the short side of the KingFisher™ 96 KF microplate (1):

Label	Number of plates
Tip comb	1

Note: The following items can be used to hold the tip comb instead of the KingFisher™ 96 KF microplate:

- Tip Comb Presenting Plate for KF 96
- Nunc™ MicroWell™ 96-Well Microplate, Flat Bottom
- Nunc™ MicroWell™ 96-Well Microplate, barcoded
- ABgene™ 96–Well Polypropylene Storage Microplate

- ABgene™ 96–Well 1.2–mL Polypropylene Deepwell Storage Plate
- Nunc™ F96 MicroWell™ Black Polystyrene Plate
- Nunc™ F96 MicroWell™ White Polystyrene Plate
- KingFisher™ Deep-Well 96 Plate

- Mark the Negative Control well on the plate.

Extract RNA—Automated method (200-µL sample input volume)

The following procedure uses components from the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit or the MagMAX™ Viral/Pathogen II Nucleic Acid Isolation Kit.

Set up the instrument (200-µL sample input volume)

1. Ensure that the KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head is set up with the KingFisher™ Flex 96 Deep-Well Heating Block.

   IMPORTANT! Failure to use the proper magnetic head and heat block results in lower yields and potential harm to the instrument.

2. Ensure that the **MVP_2Wash_200_Flex** program has been downloaded from the MagMAX™ Viral/Pathogen II Nucleic Acid Isolation Kit product page at **www.thermofisher.com** and loaded onto the instrument.

Prepare the processing plates (200-µL sample input volume)

Prepare the processing plates according to the following table. Cover the plates with a temporary seal (such as MicroAmp™ Clear Adhesive Film), then store at room temperature for up to 1 hour while you set up the sample plate.

Plate ID	Plate position	Plate type	Reagent	Volume per well
Wash 1 Plate	2	KingFisher™ Deep-Well 96 Plate	Wash Solution	500 µL
Wash 2 Plate	3		80% Ethanol	1,000 µL
Elution Plate	4		Elution Solution	50 µL
Tip Comb Plate	5	Place a KingFisher™ 96 tip comb for DW magnets in a KingFisher™ 96 KF microplate		

Note: The following items can be used to hold the tip comb instead of the KingFisher™ 96 KF microplate:

- Tip Comb Presenting Plate for KF 96
- Nunc™ MicroWell™ 96-Well Microplate, Flat Bottom
- Nunc™ MicroWell™ 96-Well Microplate, barcoded
- ABgene™ 96–Well Polypropylene Storage Microplate
- ABgene™ 96–Well 1.2–mL Polypropylene Deepwell Storage Plate
- Nunc™ F96 MicroWell™ Black Polystyrene Plate
- Nunc™ F96 MicroWell™ White Polystyrene Plate
- KingFisher™ Deep-Well 96 Plate

Prepare Binding Bead Mix (200-µL sample input volume)

Prepare the required amount of Binding Bead Mix on each day of use.

1. Vortex the Total Nucleic Acid Magnetic Beads to ensure that the bead mixture is homogeneous.
2. For the number of required reactions, prepare the Binding Bead Mix according to the following table:

Component	Volume per well[1]
Binding Solution	265 µL
Total Nucleic Acid Magnetic Beads	10 µL
Total volume per well	275 µL

[1] Include 10% overage when making the Binding Bead Mix for use with multiple reactions.

3. Mix well by inversion, then store at room temperature.

Prepare sample plate (200-µL sample input volume)

This section provides volumes for the sample plate. Your plate layout will depend on the number of samples you run.

IMPORTANT! Samples collected using the Everlywell™ COVID-19 Test Home Collection Kit have special plate layout considerations. See Appendix A, "Example extraction plate and RT-PCR reaction plate layouts (Everlywell™ COVID-19 Test Home Collection Kit samples)".

1. Add 5 µL of Proteinase K to each well in the KingFisher™ Deep-Well 96 Plate labeled "Sample Plate".
2. Add 200 µL of sample to each sample well.
   For Everlywell™ COVID-19 Test Home Collection Kit samples, not all sample wells are used. See Appendix A, "Example extraction plate and RT-PCR reaction plate layouts (Everlywell™ COVID-19 Test Home Collection Kit samples)".
3. Add 200 µL of Nuclease-free Water (not DEPC-Treated) to the Negative Control well.
   For Everlywell™ COVID-19 Test Home Collection Kit samples, prepare 2 Negative Control wells on the extraction plate if you are splitting a single extraction plate into two 96-well RT-PCR plates. See Appendix A, "Example extraction plate and RT-PCR reaction plate layouts (Everlywell™ COVID-19 Test Home Collection Kit samples)".
4. Invert the Binding Bead Mix 5 times gently to mix, then add 275 µL to each sample well and the Negative Control well in the Sample Plate.

   Note: Remix Binding Bead Mix by inversion frequently during pipetting to ensure even distribution of beads to all samples or wells. Binding Bead Mix is viscous, so pipet slowly to ensure that the correct amount is added. DO NOT reuse pipette tips to add Binding Bead Mix to the samples, as the high viscosity will cause variations in the volumes added.
5. Add 5 µL of MS2 Phage Control to each sample well and Negative Control well.

Process the samples (200-µL sample input volume)

1. Select the **MVP_2Wash_200_Flex** on the KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head.
2. Start the run, then load the prepared plates into position when prompted by the instrument.
3. After the run is complete (~22 minutes after start), immediately remove the Elution Plate from the instrument, then cover the plate with MicroAmp™ Clear Adhesive Film.

   IMPORTANT! To prevent evaporation, seal the plate containing the eluate immediately.

   The samples are eluted in 50 µL of Elution Solution (see "Prepare the processing plates (200-µL sample input volume)" on page 22).

Note:

- Significant bead carry over may adversely impact RT-PCR performance. If bead carry over is observed, repeat the test by re-extracting a new aliquot of the sample.
- To ensure reliable performance of the KingFisher™ Flex Magnetic Particle Processor, perform preventive maintenance as instructed by the manufacturer.

Place the Elution Plate on ice for immediate use in real-time RT-PCR.

Extract RNA—Automated method (400-µL sample input volume)

The following procedure uses components from the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit or the MagMAX™ Viral/Pathogen II Nucleic Acid Isolation Kit.

Set up the instrument (400-µL sample input volume)

1. Ensure that the KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head is set up with the KingFisher™ Flex 96 Deep-Well Heating Block.

   IMPORTANT! Failure to use the proper magnetic head and heat block results in lower yields and potential harm to the instrument.

2. Ensure that the **MVP_2Wash_400_Flex** program has been downloaded from the MagMAX™ Viral/Pathogen II Nucleic Acid Isolation Kit product page at **www.thermofisher.com** and loaded onto the instrument.

Prepare the processing plates (400-µL sample input volume)

Prepare the processing plates according to the following table. Cover the plates with a temporary seal (such as MicroAmp™ Clear Adhesive Film), then store at room temperature for up to 1 hour while you set up the sample plate.

Plate ID	Plate position	Plate type	Reagent	Volume per well
Wash 1 Plate	2	KingFisher™ Deep-Well 96 Plate	Wash Solution	1,000 µL
Wash 2 Plate	3		80% Ethanol	1,000 µL
Elution Plate	4		Elution Solution	50 µL
Tip Comb Plate	5	Place a KingFisher™ 96 tip comb for DW magnets in a KingFisher™ 96 KF microplate		

Note: The following items can be used to hold the tip comb instead of the KingFisher™ 96 KF microplate:

- Tip Comb Presenting Plate for KF 96
- Nunc™ MicroWell™ 96-Well Microplate, Flat Bottom
- Nunc™ MicroWell™ 96-Well Microplate, barcoded
- ABgene™ 96–Well Polypropylene Storage Microplate
- ABgene™ 96–Well 1.2–mL Polypropylene Deepwell Storage Plate
- Nunc™ F96 MicroWell™ Black Polystyrene Plate
- Nunc™ F96 MicroWell™ White Polystyrene Plate
- KingFisher™ Deep-Well 96 Plate

Prepare Binding Bead Mix (400-µL sample input volume)

Prepare the required amount of Binding Bead Mix on each day of use.

1. Vortex the Total Nucleic Acid Magnetic Beads to ensure that the bead mixture is homogeneous.
2. For the number of required reactions, prepare the Binding Bead Mix according to the following table:

Component	Volume per well[1]
Binding Solution	530 µL
Total Nucleic Acid Magnetic Beads	20 µL
Total volume per well	550 µL

[1] Include 10% overage when making the Binding Bead Mix for use with multiple reactions.

3. Mix well by inversion, then store at room temperature.

Prepare sample plate (400-µL sample input volume)

This section provides volumes for the sample plate. Your plate layout will depend on the number of samples you run.

IMPORTANT! Samples collected using the Everlywell™ COVID-19 Test Home Collection Kit have special plate layout considerations. See Appendix A, "Example extraction plate and RT-PCR reaction plate layouts (Everlywell™ COVID-19 Test Home Collection Kit samples)".

1. Add 10 µL of Proteinase K to each well in the KingFisher™ Deep-Well 96 Plate labeled "Sample Plate".
2. Add 400 µL of sample to each sample well.
   For Everlywell™ COVID-19 Test Home Collection Kit samples, not all sample wells may be used. See Appendix A, "Example extraction plate and RT-PCR reaction plate layouts (Everlywell™ COVID-19 Test Home Collection Kit samples)".
3. Add 400 µL of Nuclease-free Water (not DEPC-Treated) to the Negative Control well.
   For Everlywell™ COVID-19 Test Home Collection Kit samples, prepare 2 Negative Control wells on the extraction plate if you are splitting a single extraction plate into two 96-well RT-PCR plates. See Appendix A, "Example extraction plate and RT-PCR reaction plate layouts (Everlywell™ COVID-19 Test Home Collection Kit samples)".
4. Invert the Binding Bead Mix 5 times gently to mix, then add 550 µL to each sample well and the Negative Control well in the Sample Plate.

   Note: Remix the Binding Bead Mix by inversion frequently during pipetting to ensure even distribution of beads to all samples or wells. The Binding Bead Mix is viscous, so pipet slowly to ensure that the correct amount is added. DO NOT reuse pipette tips to add Binding Bead Mix to the samples, as the high viscosity will cause variations in the volumes added.
5. Add 10 µL of MS2 Phage Control to each sample well and to the Negative Control well.

Process the samples (400-µL sample input volume)

1. Select the **MVP_2Wash_400_Flex** on the KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head.
2. Start the run, then load the prepared plates into position when prompted by the instrument.
3. After the run is complete (~24 minutes after start), immediately remove the Elution Plate from the instrument, then cover the plate with MicroAmp™ Clear Adhesive Film.

   IMPORTANT! To prevent evaporation, seal the plate containing the eluate immediately.

   The samples are eluted in 50 µL of Elution Solution (see "Prepare the processing plates (400-µL sample input volume)" on page 25).

Note:

- Significant bead carry over may adversely impact RT-PCR performance. If bead carry over is observed, repeat the test by re-extracting a new aliquot of the sample.
- To ensure reliable performance of the KingFisher™ Flex Magnetic Particle Processor, perform preventive maintenance as instructed by the manufacturer.

Place the Elution Plate on ice for immediate use in real-time RT-PCR.

3 Extract RNA (manual method)

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- Extract RNA—Manual method (200-µL sample input volume) 28
- Extract RNA—Manual method (400-µL sample input volume) 31

Manual RNA extraction can be performed from a sample input volume of 200 µL or 400 µL using either the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit or the MagMAX™ Viral/Pathogen II Nucleic Acid Isolation Kit.

Before you begin

Note: During the wash steps, the Wash Solution may develop inert white or brown particulates that float in solution. This is not a cause for concern and does not negatively affect performance.

- Determine the number of required reactions based on the number of patient samples to be processed, plus one Negative Control per plate.
- Prepare fresh 80% Ethanol using Ethanol, Absolute, Molecular Biology Grade and Nuclease-free Water (not DEPC-Treated) for the required number of reactions, plus 10% overage.

Sample input volume	Volume of 80% Ethanol per reaction
200 µL	0.75 mL
400 µL	1.5 mL

- Mark the Negative Control well on the plate.

Extract RNA—Manual method (200-µL sample input volume)

The following procedure uses components from the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit or the MagMAX™ Viral/Pathogen II Nucleic Acid Isolation Kit.

Prepare Binding Bead Mix (200-µL sample input volume)

Prepare the required amount of Binding Bead Mix on each day of use.

1. Vortex the Total Nucleic Acid Magnetic Beads to ensure that the bead mixture is homogeneous.
2. For the number of required reactions, prepare the Binding Bead Mix according to the following table:

Component	Volume per well[1]
Binding Solution	265 µL
Total Nucleic Acid Magnetic Beads	10 µL
Total volume per well	275 µL

[1] Include 10% overage when making the Binding Bead Mix for use with multiple reactions.

3. Mix well by inversion, then store at room temperature.

Digest with Proteinase K (200-µL sample input volume)

This section provides volumes for the sample plate. Your plate layout will depend on the number of samples you run.

IMPORTANT! Samples collected using the Everlywell™ COVID-19 Test Home Collection Kit have special plate layout considerations. See Appendix A, "Example extraction plate and RT-PCR reaction plate layouts (Everlywell™ COVID-19 Test Home Collection Kit samples)".

---

1. Add 5 µL of Proteinase K to each well of a KingFisher™ Deep-Well 96 Plate.
2. Add 200 µL of sample to each sample well.

   For Everlywell™ COVID-19 Test Home Collection Kit samples, not all sample wells may be used. See Appendix A, "Example extraction plate and RT-PCR reaction plate layouts (Everlywell™ COVID-19 Test Home Collection Kit samples)".
3. Add 200 µL of Nuclease-free Water (not DEPC-Treated) to the Negative Control well.

   For Everlywell™ COVID-19 Test Home Collection Kit samples, prepare 2 Negative Control wells on the extraction plate if you are splitting a single extraction plate into two 96-well RT-PCR plates. See Appendix A, "Example extraction plate and RT-PCR reaction plate layouts (Everlywell™ COVID-19 Test Home Collection Kit samples)".
4. Invert the Binding Bead Mix 5 times gently to mix, then add 275 µL to each sample well and Negative Control well.

   Note: Remix the Binding Bead Mix by inversion frequently during pipetting to ensure even distribution of beads to all samples or wells. The Binding Bead Mix is viscous, so pipet slowly to ensure that the correct amount is added. DO NOT reuse pipette tips to add Binding Bead Mix to the samples, as the high viscosity will cause variations in the volumes added.

   ---
5. Add 5 µL of MS2 Phage Control to each sample well and to the Negative Control well.

6. Seal the plate with MicroAmp™ Clear Adhesive Film, then shake the sealed plate at 1,050 rpm for 2 minutes.

7. Incubate the sealed plate at 65°C for 5 minutes (ensure the bottom of the plate is uncovered), then shake the plate at 1,050 rpm for 5 minutes.

8. Place the sealed plate on the magnetic stand for 10 minutes or until all of the beads have collected.

Wash the beads (200-µL sample input volume)

1. Keeping the plate on the magnet, carefully remove the cover, then discard the supernatant from each well.

   IMPORTANT! Avoid disturbing the beads.

2. Remove the plate from the magnetic stand, then add 500 µL of Wash Solution to each sample.

3. Reseal the plate, then shake at 1,050 rpm for 1 minute.

4. Place the plate back on the magnetic stand for 2 minutes, or until all the beads have collected.

5. Keeping the plate on the magnet, carefully remove the cover, then discard the supernatant from each well.

   IMPORTANT! Avoid disturbing the beads.

6. Repeat step 2 to step 5 using 500 µL of 80% Ethanol.

7. Repeat step 2 to step 5 using 250 µL of 80% Ethanol.

8. Dry the beads by shaking the plate (uncovered) at 1,050 rpm for 2 minutes.

Elute the nucleic acid (200-µL sample input volume)

1. Add 50 µL of Elution Solution to each sample, then seal the plate with MicroAmp™ Clear Adhesive Film.

2. Shake the sealed plate at 1,050 rpm for 5 minutes.

3. Place the plate in an incubator at 65°C for 10 minutes.

4. Remove the plate from the incubator, then shake the plate at 1,050 rpm for 5 minutes.

5. Place the sealed plate on the magnetic stand for 3 minutes or until clear to collect the beads against the magnets.

6. Keeping the plate on the magnet, carefully remove the seal, transfer the eluates to a fresh standard (not deep-well) 96-well plate, then seal the plate with MicroAmp™ Clear Adhesive Film.

   IMPORTANT! To prevent evaporation, seal the plate containing the eluate immediately after the transfers are complete.



Note: Significant bead carry over may adversely impact RT-PCR performance. If bead carry over is observed, repeat the test by re-extracting a new aliquot of the sample.

Place the plate on ice for immediate use in real-time RT-PCR.

Extract RNA—Manual method (400-µL sample input volume)

The following procedure uses components from the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit or the MagMAX™ Viral/Pathogen II Nucleic Acid Isolation Kit.

Prepare Binding Bead Mix (400-µL sample input volume)

Prepare the required amount of Binding Bead Mix on each day of use.

1. Vortex the Total Nucleic Acid Magnetic Beads to ensure that the bead mixture is homogeneous.
2. For the number of required reactions, prepare the Binding Bead Mix according to the following table:

Component	Volume per well[1]
Binding Solution	530 µL
Total Nucleic Acid Magnetic Beads	20 µL
Total volume per well	550 µL

[1] Include 10% overage when making the Binding Bead Mix for use with multiple reactions.

3. Mix well by inversion, then store at room temperature.

Digest with Proteinase K (400-µL sample input volume)

This section provides volumes for the sample plate. Your plate layout will depend on the number of samples you run.

IMPORTANT! Samples collected using the Everlywell™ COVID-19 Test Home Collection Kit have special plate layout considerations. See Appendix A, "Example extraction plate and RT-PCR reaction plate layouts (Everlywell™ COVID-19 Test Home Collection Kit samples)".

1. Add 10 µL of Proteinase K to each well of a KingFisher™ Deep-Well 96 Plate.
2. Add 400 µL of sample to each sample well.

   For Everlywell™ COVID-19 Test Home Collection Kit samples, not all sample wells may be used. See Appendix A, "Example extraction plate and RT-PCR reaction plate layouts (Everlywell™ COVID-19 Test Home Collection Kit samples)".

3. Add 400 µL of Nuclease-free Water (not DEPC-Treated) to the Negative Control well.

   For Everlywell™ COVID-19 Test Home Collection Kit samples, prepare 2 Negative Control wells on the extraction plate if you are splitting a single extraction plate into two 96-well RT-PCR plates. See Appendix A, "Example extraction plate and RT-PCR reaction plate layouts (Everlywell™ COVID-19 Test Home Collection Kit samples)".

4. Invert the Binding Bead Mix 5 times gently to mix, then add 550 µL to each sample well and Negative Control well.

   Note: Remix the Binding Bead Mix by inversion frequently during pipetting to ensure even distribution of beads to all samples or wells. The Binding Bead Mix is viscous, so pipet slowly to ensure that the correct amount is added. DO NOT reuse pipette tips to add Binding Bead Mix to the samples, as the high viscosity will cause variations in the volumes added.

5. Add 10 µL of MS2 Phage Control to each sample well and to the Negative Control well.

6. Seal the plate with MicroAmp™ Clear Adhesive Film, then shake the sealed plate at 1,050 rpm for 2 minutes.

7. Incubate the sealed plate at 65°C for 5 minutes (ensure the bottom of the plate is uncovered), then shake the plate at 1,050 rpm for 5 minutes.

8. Place the sealed plate on the magnetic stand for 10 minutes or until all of the beads have collected.

Wash the beads (400-µL sample input volume)

1. Keeping the plate on the magnet, carefully remove the cover, then discard the supernatant from each well.

   IMPORTANT! Avoid disturbing the beads.

2. Remove the plate from the magnetic stand, then add 1 mL of Wash Solution to each sample.

3. Reseal the plate, then shake at 1,050 rpm for 1 minute.

4. Place the plate back on the magnetic stand for 2 minutes, or until all the beads have collected.

5. Keeping the plate on the magnet, carefully remove the cover, then discard the supernatant from each well.

   IMPORTANT! Avoid disturbing the beads.

6. Repeat step 2 to step 5 using 1 mL of 80% Ethanol.

7. Repeat step 2 to step 5 using 500 µL of 80% Ethanol.

8. Dry the beads by shaking the plate (uncovered) at 1,050 rpm for 2 minutes.



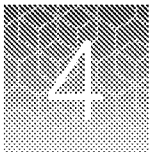
Elute the nucleic acid (400-µL sample input volume)

1. Add 50 µL of Elution Solution to each sample, then seal the plate with MicroAmp™ Clear Adhesive Film.
2. Shake the sealed plate at 1,050 rpm for 5 minutes.
3. Place the plate in an incubator at 65°C for 10 minutes.
4. Remove the plate from the incubator, then shake the plate at 1,050 rpm for 5 minutes.
5. Place the sealed plate on the magnetic stand for 3 minutes or until clear to collect the beads against the magnets.
6. Keeping the plate on the magnet, carefully remove the seal, transfer the eluates to a fresh standard (not deep-well) 96-well plate, then seal the plate with MicroAmp™ Clear Adhesive Film.

   IMPORTANT! To prevent evaporation, seal the plate containing the eluate immediately after the transfers are complete.

Note: Significant bead carry over may adversely impact RT-PCR performance. If bead carry over is observed, repeat the test by re-extracting a new aliquot of the sample.

Place the plate on ice for immediate use in real-time RT-PCR.



Prepare RT-PCR reactions—COVID-19 assay only

- Protocols in this chapter 34
- Guidelines for RT-PCR 35
- Prepare the RT-PCR reactions (200-µL sample input, 96-well reaction plate, COVID-19 assay only) 35
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Protocols in this chapter

This chapter covers preparation of RT-PCR reaction plates for samples that were collected using methods other than the Everlywell™ COVID-19 Test Home Collection Kit. The RT-PCR reactions in this chapter use the COVID-19 Real Time PCR Assay Multiplex or COVID-19 Assay Multiplex Advanced, but do not include the RNase P assay.

Note: For samples collected using the Everlywell™ COVID-19 Test Home Collection Kit, see Chapter 5, "Prepare RT-PCR reactions—COVID-19 assay plus RNase P assay (Everlywell™ COVID-19 Test Home Collection Kit)".

IMPORTANT! Do not combine samples collected using the Everlywell™ COVID-19 Test Home Collection Kit with samples collected via other methods on the same RT-PCR plate. The plate setup and software template files are different.

In this chapter, select the appropriate RT-PCR preparation procedure based on the following criteria:

- The original sample input volume used for RNA extraction (200 µL or 400 µL)
- The size of the RT-PCR reaction plate (96–well or a 384–well)
- The volume of purified sample RNA that is added to the reaction

Guidelines for RT-PCR

IMPORTANT!

- For each RT-PCR reaction plate, include the following controls:
  - One Positive Control
  - One Negative Control from each extraction run.
    For example, if RNA samples from 4 extraction runs are combined on one 384-well RT-PCR reaction plate, then 4 Negative Control wells must be run on that 384-well reaction plate.
- Prepare the RT-PCR reaction plate on ice and keep it on ice until it is loaded into the real-time PCR instrument.
- Run the plate immediately after preparation. Failure to do so could result in degraded RNA samples.
- To prevent contamination, prepare reagents in a PCR workstation or equivalent amplicon-free area. Do not use the same pipette for controls and RNA samples, and always use aerosol barrier pipette tips.
- Maintain an RNase-free environment.
- Protect assays from light.
- Keep RNA samples and components on ice during use.

Prepare the RT-PCR reactions (200-µL sample input, 96-well reaction plate, COVID-19 assay only)

Use this procedure under the following conditions:

- Original sample input volume of 200 µL was used for extraction
- Instrument is compatible with 96-well RT-PCR reaction plates
- Sample was not collected with the Everlywell™ COVID-19 Test Home Collection Kit (no RNase P assay required)

1. If frozen, thaw the reagents on ice.
2. Gently vortex the reagents, then centrifuge briefly to collect liquid at the bottom of the tube.
3. Dilute TaqPath™ COVID-19 Control (1×10^4 copies/µL) to a working stock of 25 copies/µL:
   a. Pipet 98 µL of TaqPath™ COVID-19 Control Dilution Buffer into a microcentrifuge tube, then add 2 µL of TaqPath™ COVID-19 Control. Mix well, then centrifuge briefly.
   b. Pipet 87.5 µL of TaqPath™ COVID-19 Control Dilution Buffer into a second microcentrifuge tube, then add 12.5 µL of the dilution created in substep 3a. Mix well, then centrifuge briefly.

Note: The TaqPath™ COVID-19 Control does not contain the MS2 template.

4. Prepare the Reaction Mix:
   a. For each run, combine the following components sufficient for the number of RNA samples to be tested plus one Positive Control and one Negative Control.

All volumes include 10% overage for pipette error.

IMPORTANT! The volumes in this table assume that you extracted sample RNA using an original sample input volume of 200 µL.

Component	Volume per RNA sample or control	Volume for *n* RNA samples plus 2 controls	Volume for 94 RNA Samples plus 2 controls
TaqPath™ 1-Step Multiplex Master Mix (No ROX™) (4X)	6.25 µL	6.875 x (*n* + 2) µL	660 µL
COVID-19 Real Time PCR Assay Multiplex	1.25 µL	1.375 x (*n* + 2) µL	132 µL
Nuclease-free Water	7.50 µL	8.25 x (*n* + 2) µL	792 µL
Total Reaction Mix volume	**15.0 µL**	—	**1584 µL**

5. Set up the reaction plate:
   a. Pipette 15.0 µL of the Reaction Mix prepared in step 4 into each well of a MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL or a MicroAmp™ Optical 96-Well Reaction Plate with Barcode, 0.2 mL.

      Plates without a barcode can be used (see "Required materials not supplied" on page 11).

   b. Gently vortex the sealed plate containing the purified sample RNA and Negative Control from the RNA extraction procedure, then centrifuge briefly to collect liquid at the bottom of the plate.

   c. Unseal the plate containing the purified sample RNA and Negative Control from the RNA extraction procedure. Add either sample RNA, Negative Control, or Positive Control to each well of the reaction plate according to Table 3 on page 37.

   d. Seal the plate thoroughly with MicroAmp™ Optical Adhesive Film.

      IMPORTANT! When applying the MicroAmp™ Optical Adhesive Film, ensure that pressure is applied across the entire plate and that there is a tight seal across every individual well. Failure to do so runs the risk of an improperly sealed well, leading to potential well-to-well contamination during vortexing and evaporation during PCR.

   e. Vortex the plate at the highest setting speed for 10–30 seconds with medium pressure. Move the plate around to ensure equal contact on the vortex mixer platform.

      IMPORTANT! Vortex for 10–30 seconds to ensure proper mixing. Failure to do so might result in false classification of samples.



f. Centrifuge the reaction plate for 1–2 minutes at ≥650 × *g* (≥650 RCF) to remove bubbles and to collect the liquid at the bottom of the reaction plate.

Table 3 Reaction plate volumes

Component	Volume per reaction		
	RNA Sample reaction	Positive Control reaction	Negative Control reaction
Reaction Mix	15.0 µL	15.0 µL	15.0 µL
Purified sample RNA (from RNA extraction)	10.0 µL	—	—
Positive Control (diluted TaqPath™ COVID-19 Control, from step 3)	—	2.0 µL	—
Nuclease-free Water	—	8.0 µL	—
Purified Negative Control (from RNA extraction)	—	—	10.0 µL
Total volume	**25.0 µL**	**25.0 µL**	**25.0 µL**

Prepare the RT-PCR reactions (200-µL sample input, 384-well reaction plate, COVID-19 assay only)

Use this procedure under the following conditions:

- Original sample input volume of 200 µL was used for extraction
- Instrument is compatible with 384-well RT-PCR reaction plates
- Sample was not collected with the Everlywell™ COVID-19 Test Home Collection Kit (no RNase P assay required)

1. If frozen, thaw the reagents on ice.

2. Gently vortex the reagents, then centrifuge briefly to collect liquid at the bottom of the tube.

3. Dilute TaqPath™ COVID-19 Control (1×10^4 copies/µL) to a working stock of 25 copies/µL:
   a. Pipet 98 µL of TaqPath™ COVID-19 Control Dilution Buffer into a microcentrifuge tube, then add 2 µL of TaqPath™ COVID-19 Control. Mix well, then centrifuge briefly.

   b. Pipet 87.5 µL of TaqPath™ COVID-19 Control Dilution Buffer into a second microcentrifuge tube, then add 12.5 µL of the dilution created in substep 3a. Mix well, then centrifuge briefly.

Note: The TaqPath™ COVID-19 Control does not contain the MS2 template.

4. Prepare the Reaction Mix.
   a. For each run, combine the following components sufficient for the number of RNA samples, plus one Positive Control per 384-well real-time RT-PCR plate, and one Negative Control from each extraction run.

      For example, if RNA samples from 4 extraction runs are being combined on one 384-well real-time RT-PCR plate, then 4 Negative Control wells need to be run on that 384-well real-time RT-PCR plate.

All volumes include 10% overage for pipette error.

IMPORTANT! The volumes in this table assume that you extracted sample RNA using an original sample input volume of 200 µL.

Component	Volume per RNA Sample or control	Volume for *n* RNA Samples plus *y* Negative Controls plus 1 Positive Control	Volume for 379 RNA Samples plus 4 Negative Controls plus 1 Positive Control
TaqPath™ 1-Step Multiplex Master Mix (No ROX™) (4X)	5.00 µL	5.50 x (*n* + *y* + 1) µL	2112.0 µL
COVID-19 Real Time PCR Assay Multiplex	1.00 µL	1.10 x (*n* + *y* + 1) µL	422.4 µL
Nuclease-free Water	4.00 µL	4.40 x (*n* + *y* + 1) µL	1690.0 µL
Total Reaction Mix volume	**10.0 µL**	—	**4224.4 µL**

5. Set up the reaction plate:
   a. Pipette 10.0 µL of the Reaction Mix prepared in step 4 into each well of a MicroAmp™ Optical 384-Well Reaction Plate with Barcode.

      Plates without a barcode can be used (see "Required materials not supplied" on page 11).

   b. Gently vortex the sealed plate containing the purified sample RNA and Negative Control from the RNA extraction procedure, then centrifuge briefly to collect liquid at the bottom of the plate.

   c. Unseal the plate containing the purified sample RNA and Negative Control from the RNA extraction procedure. Add either sample RNA, Negative Control, or Positive Control to each well of the reaction plate according to Table 4 on page 39.

      IMPORTANT! To prevent sample contamination, unseal one extraction plate at a time, then reseal it after adding the samples to the RT-PCR reaction plate.

   d. Seal the plate thoroughly with MicroAmp™ Optical Adhesive Film.

      IMPORTANT! When applying the MicroAmp™ Optical Adhesive Film, ensure that pressure is applied across the entire plate and that there is a tight seal across every individual well. Failure to do so runs the risk of an improperly sealed well, leading to potential well-to-well contamination during vortexing and evaporation during PCR.

e. Vortex the plate at the highest setting speed for 10–30 seconds with medium pressure. Move the plate around to ensure equal contact on the vortex mixer platform.

IMPORTANT! Vortex for 10–30 seconds to ensure proper mixing. Failure to do so might result in false classification of samples.

f. Centrifuge the reaction plate for 1–2 minutes at ≥650 × *g* (≥650 RCF) to remove bubbles and to collect the liquid at the bottom of the reaction plate.

Table 4 Reaction plate volumes

Component	Volume per reaction		
	RNA Sample reaction	Positive Control reaction	Negative Control reaction
Reaction Mix	10.0 µL	10.0 µL	10.0 µL
Purified sample RNA (from RNA extraction)	10.0 µL	—	—
Positive Control (diluted TaqPath™ COVID-19 Control from step 3)	—	2.0 µL	—
Nuclease-free Water	—	8.0 µL	—
Purified Negative Control (from RNA extraction)	—	—	10.0 µL
Total volume	**20.0 µL**	**20.0 µL**	**20.0 µL**

Prepare the RT-PCR reactions (400-µL sample input, 96-well reaction plate, COVID-19 assay only)

Use this procedure under the following conditions:

- Original sample input volume of 400 µL was used for extraction
- Instrument is compatible with 96-well RT-PCR reaction plates
- Sample was not collected with the Everlywell™ COVID-19 Test Home Collection Kit (no RNase P assay required)

1. If frozen, thaw the reagents on ice.

2. Gently vortex the reagents, then centrifuge briefly to collect liquid at the bottom of the tube.

3. Dilute TaqPath™ COVID-19 Control (1×10^4 copies/µL) to a working stock of 25 copies/µL:
   a. Pipet 98 µL of TaqPath™ COVID-19 Control Dilution Buffer into a microcentrifuge tube, then add 2 µL of TaqPath™ COVID-19 Control. Mix well, then centrifuge briefly.

   b. Pipet 87.5 µL of TaqPath™ COVID-19 Control Dilution Buffer into a second microcentrifuge tube, then add 12.5 µL of the dilution created in substep 3a. Mix well, then centrifuge briefly.

Note: The TaqPath™ COVID-19 Control does not contain the MS2 template.

4. Prepare the Reaction Mix:
   a. For each run, combine the following components sufficient for the number of RNA samples to be tested plus one Positive Control and one Negative Control.

All volumes include 10% overage for pipette error.

IMPORTANT! The volumes in this table assume that you extracted sample RNA using an original sample input volume of 400 µL.

Component	Volume per RNA Sample or control	Volume for *n* RNA Samples plus 2 controls	Volume for 94 RNA Samples plus 2 controls
TaqPath™ 1-Step Multiplex Master Mix (No ROX™) (4X)	6.25 µL	6.875 x (*n* + 2) µL	660 µL
COVID-19 Real Time PCR Assay Multiplex	1.25 µL	1.375 x (*n* + 2) µL	132 µL
Nuclease-free Water	12.50 µL	13.75 x (*n* + 2) µL	1320 µL
Total Reaction Mix volume	**20.0 µL**	—	**2112 µL**

5. Set up the reaction plate:
   a. Pipette 20.0 µL of the Reaction Mix prepared in step 4 into each well of a MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL or a MicroAmp™ Optical 96-Well Reaction Plate with Barcode, 0.2 mL.

      Plates without a barcode can be used (see "Required materials not supplied" on page 11).

   b. Gently vortex the sealed plate containing the purified sample RNA and Negative Control from the RNA extraction procedure, then centrifuge briefly to collect liquid at the bottom of the plate.

   c. Unseal the plate containing the purified sample RNA and Negative Control from the RNA extraction procedure. Add either sample RNA, Negative Control, or Positive Control to each well of the reaction plate according to Table 5 on page 41.

   d. Seal the plate thoroughly with MicroAmp™ Optical Adhesive Film.

      IMPORTANT! When applying the MicroAmp™ Optical Adhesive Film, ensure that pressure is applied across the entire plate and that there is a tight seal across every individual well. Failure to do so runs the risk of an improperly sealed well, leading to potential well-to-well contamination during vortexing and evaporation during PCR.

   e. Vortex the plate at the highest setting speed for 10–30 seconds with medium pressure. Move the plate around to ensure equal contact on the vortex mixer platform.

      IMPORTANT! Vortex for 10–30 seconds to ensure proper mixing. Failure to do so might result in false classification of samples.



f. Centrifuge the reaction plate for 1–2 minutes at ≥650 × *g* (≥650 RCF) to remove bubbles and to collect the liquid at the bottom of the reaction plate.

Table 5 Reaction plate volumes

Component	Volume per reaction		
	RNA Sample reaction	Positive Control reaction	Negative Control reaction
Reaction Mix	20.0 µL	20.0 µL	20.0 µL
Purified sample RNA (from RNA extraction)	5.0 µL	—	—
Positive Control (diluted TaqPath™ COVID-19 Control from step 3)	—	2.0 µL	—
Nuclease-free Water	—	3.0 µL	—
Purified Negative Control (from RNA extraction)	—	—	5.0 µL
Total volume	**25.0 µL**	**25.0 µL**	**25.0 µL**

Prepare the RT-PCR reactions (400-µL sample input, 384-well reaction plate, COVID-19 assay only)

Use this procedure under the following conditions:

- Original sample input volume of 400 µL was used for extraction
- Instrument is compatible with 384-well RT-PCR reaction plates
- Sample was not collected with the Everlywell™ COVID-19 Test Home Collection Kit (no RNase P assay required)

1. If frozen, thaw the reagents on ice.
2. Gently vortex the reagents, then centrifuge briefly to collect liquid at the bottom of the tube.
3. Dilute TaqPath™ COVID-19 Control (1×10^4 copies/µL) to a working stock of 25 copies/µL:
   a. Pipet 98 µL of TaqPath™ COVID-19 Control Dilution Buffer into a microcentrifuge tube, then add 2 µL of TaqPath™ COVID-19 Control. Mix well, then centrifuge briefly.
   b. Pipet 87.5 µL of TaqPath™ COVID-19 Control Dilution Buffer into a second microcentrifuge tube, then add 12.5 µL of the dilution created in substep 3a. Mix well, then centrifuge briefly.

Note: The TaqPath™ COVID-19 Control does not contain the MS2 template.

4. Prepare the Reaction Mix.
   a. For each run, combine the following components sufficient for the number of RNA samples, plus one Positive Control per 384-well real-time RT-PCR plate, and one Negative Control from each extraction run.

      For example, if RNA samples from 4 extraction runs are being combined on one 384-well real-time RT-PCR plate, then 4 Negative Control wells need to be run on that 384-well real-time RT-PCR plate.

All volumes include 10% overage for pipette error.

IMPORTANT! The volumes in this table assume that you extracted sample RNA using an original sample input volume of 400 µL.

Component	Volume per RNA Sample or control	Volume for *n* RNA Samples plus *y* Negative Controls plus 1 Positive Control	Volume for 379 RNA Samples plus 4 Negative Controls plus 1 Positive Control
TaqPath™ 1-Step Multiplex Master Mix (No ROX™) (4X)	5.00 µL	5.50 x (*n* + *y* + 1) µL	2112.0 µL
COVID-19 Real Time PCR Assay Multiplex	1.00 µL	1.10 x (*n* + *y* + 1) µL	422.4 µL
Nuclease-free Water	9.00 µL	9.90 x (*n* + *y* + 1) µL	3802.0 µL
Total Reaction Mix volume	**15.0 µL**	—	**6336.4 µL**

5. Set up the reaction plate:
   a. Pipette 15.0 µL of the Reaction Mix prepared in step 4 into each well of a MicroAmp™ Optical 384-Well Reaction Plate with Barcode.

      Plates without a barcode can be used (see "Required materials not supplied" on page 11).

   b. Gently vortex the sealed plate containing the purified sample RNA and Negative Control from the RNA extraction procedure, then centrifuge briefly to collect liquid at the bottom of the plate.

   c. Unseal the plate containing the purified sample RNA and Negative Control from the RNA extraction procedure. Add either sample RNA, Negative Control, or Positive Control to each well of the reaction plate according to Table 6 on page 43.

      IMPORTANT! To prevent sample contamination, unseal one extraction plate at a time, then reseal it after adding the samples to the RT-PCR reaction plate.

   d. Seal the plate thoroughly with MicroAmp™ Optical Adhesive Film.

      IMPORTANT! When applying the MicroAmp™ Optical Adhesive Film, ensure that pressure is applied across the entire plate and that there is a tight seal across every individual well. Failure to do so runs the risk of an improperly sealed well, leading to potential well-to-well contamination during vortexing and evaporation during PCR.

e. Vortex the plate at the highest setting speed for 10–30 seconds with medium pressure. Move the plate around to ensure equal contact on the vortex mixer platform.

IMPORTANT! Vortex for 10–30 seconds to ensure proper mixing. Failure to do so might result in false classification of samples.

f. Centrifuge the reaction plate for 1–2 minutes at ≥650 × *g* (≥650 RCF) to remove bubbles and to collect the liquid at the bottom of the reaction plate.

Table 6 Reaction plate volumes

Component	Volume per reaction		
	RNA Sample reaction	Positive Control reaction	Negative Control reaction
Reaction Mix	15.0 µL	15.0 µL	15.0 µL
Purified sample RNA (from RNA extraction)	5.0 µL	—	—
Positive Control (diluted TaqPath™ COVID-19 Control from step 3)	—	2.0 µL	—
Nuclease-free Water	—	3.0 µL	—
Purified Negative Control (from RNA extraction)	—	—	5.0 µL
Total volume	**20.0 µL**	**20.0 µL**	**20.0 µL**

Prepare RT-PCR reactions with 17.5 µL of purified sample RNA (400-µL sample input, 96-well reaction plate, COVID-19 assay only)

IMPORTANT!

- The volumes provided in the TaqPath™ COVID-19 Combo Kit Advanced (Cat. No. A47813) have been optimized for this procedure.
- The sample volumes used in this procedure are not compatible with samples collected using the Everlywell™ COVID-19 Test Home Collection Kit.

Use this procedure to prepare RT-PCR reactions under the following conditions.

- Original sample input volume of 400 µL was used for extraction
- 17.5 µL of purified sample RNA is added to the reaction
- Instrument is compatible with 96-well RT-PCR reaction plates
- Sample was not collected with the Everlywell™ COVID-19 Test Home Collection Kit (no RNase P assay required)



1. If frozen, thaw the reagents on ice.

2. Gently vortex the reagents, then centrifuge briefly to collect liquid at the bottom of the tube.

3. Dilute TaqPath™ COVID-19 Control (1×10^4 copies/µL) to a working stock of 25 copies/µL:
   a. Pipet 98 µL of TaqPath™ COVID-19 Control Dilution Buffer into a microcentrifuge tube, then add 2 µL of TaqPath™ COVID-19 Control. Mix well, then centrifuge briefly.

   b. Pipet 87.5 µL of TaqPath™ COVID-19 Control Dilution Buffer into a second microcentrifuge tube, then add 12.5 µL of the dilution created in substep 3a. Mix well, then centrifuge briefly.

Note: The TaqPath™ COVID-19 Control does not contain the MS2 template.

4. Prepare the Reaction Mix:
   a. For each run, combine the following components sufficient for the number of RNA samples to be tested plus one Positive Control and one Negative Control.

All volumes include 10% overage for pipette error.

IMPORTANT! The volumes in this table assume that you extracted sample RNA using an original sample input volume of 400 µL.

Component	Volume per RNA Sample or control	Volume for *n* RNA Samples plus 2 controls	Volume for 94 RNA Samples plus 2 controls
TaqPath™ 1-Step Multiplex Master Mix (No ROX™) (4X)	6.25 µL	6.875 x (*n* + 2) µL	660 µL
COVID-19 Assay Multiplex Advanced	1.25 µL	1.375 x (*n* + 2) µL	132 µL
Total Reaction Mix volume	**7.5 µL**	—	**792 µL**

5. Set up the reaction plate:
   a. Pipette 7.5 µL of the Reaction Mix prepared in step 4 into each well of a MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL or a MicroAmp™ Optical 96-Well Reaction Plate with Barcode, 0.2 mL.

   Plates without a barcode can be used (see "Required materials not supplied" on page 11).

   b. Gently vortex the sealed plate containing the purified sample RNA and Negative Control from the RNA extraction procedure, then centrifuge briefly to collect liquid at the bottom of the plate.

   c. Unseal the plate containing the purified sample RNA and Negative Control from the RNA extraction procedure. Add either sample RNA, Negative Control, or Positive Control to each well of the reaction plate according to Table 7 on page 45.



d. Seal the plate thoroughly with MicroAmp™ Optical Adhesive Film.

IMPORTANT! When applying the MicroAmp™ Optical Adhesive Film, ensure that pressure is applied across the entire plate and that there is a tight seal across every individual well. Failure to do so runs the risk of an improperly sealed well, leading to potential well-to-well contamination during vortexing and evaporation during PCR.

e. Vortex the plate at the highest setting speed for 10–30 seconds with medium pressure. Move the plate around to ensure equal contact on the vortex mixer platform.

IMPORTANT! Vortex for 10–30 seconds to ensure proper mixing. Failure to do so might result in false classification of samples.

f. Centrifuge the reaction plate for 1–2 minutes at ≥650 × *g* (≥650 RCF) to remove bubbles and to collect the liquid at the bottom of the reaction plate.

Table 7 Reaction plate volumes

Component	Volume per reaction		
	RNA Sample reaction	Positive Control reaction	Negative Control reaction
Reaction Mix	7.5 µL	7.5 µL	7.5 µL
Purified sample RNA (from RNA extraction)	17.5 µL	—	—
Positive Control (diluted TaqPath™ COVID-19 Control from step 3)	—	2.0 µL	—
Nuclease-free Water	—	15.5 µL	—
Purified Negative Control (from RNA extraction)	—	—	17.5 µL
Total volume	**25.0 µL**	**25.0 µL**	**25.0 µL**



Prepare RT-PCR reactions with 14.0 µL of purified sample RNA (400-µL sample input, 384-well reaction plate, COVID-19 assay only)

IMPORTANT!

- The volumes provided in the TaqPath™ COVID-19 Combo Kit Advanced (Cat. No. A47813) have been optimized for this procedure.
- The sample volumes used in this procedure are not compatible with samples collected using the Everlywell™ COVID-19 Test Home Collection Kit.

Use this procedure to prepare RT-PCR reactions under the following conditions

- Original sample input volume of 400 µL was used for extraction
- 14.0 µL of purified sample RNA is added to the reaction
- Instrument is compatible with 384-well RT-PCR reaction plates
- Sample was not collected with the Everlywell™ COVID-19 Test Home Collection Kit (no RNase P assay required)

1. If frozen, thaw the reagents on ice.

2. Gently vortex the reagents, then centrifuge briefly to collect liquid at the bottom of the tube.

3. Dilute TaqPath™ COVID-19 Control (1×10^4 copies/µL) to a working stock of 25 copies/µL:
   a. Pipet 98 µL of TaqPath™ COVID-19 Control Dilution Buffer into a microcentrifuge tube, then add 2 µL of TaqPath™ COVID-19 Control. Mix well, then centrifuge briefly.

   b. Pipet 87.5 µL of TaqPath™ COVID-19 Control Dilution Buffer into a second microcentrifuge tube, then add 12.5 µL of the dilution created in substep 3a. Mix well, then centrifuge briefly.

Note: The TaqPath™ COVID-19 Control does not contain the MS2 template.

4. Prepare the Reaction Mix.
   a. For each run, combine the following components sufficient for the number of RNA samples, plus one Positive Control per 384-well real-time RT-PCR plate, and one Negative Control from each extraction run.

   For example, if RNA samples from 4 extraction runs are being combined on one 384-well real-time RT-PCR plate, then 4 Negative Control wells need to be run on that 384-well real-time RT-PCR plate.



All volumes include 10% overage for pipette error.

IMPORTANT! The volumes in this table assume that you extracted sample RNA using an original sample input volume of 400 µL.

Component	Volume per RNA Sample or control	Volume for *n* RNA Samples plus *y* Negative Controls plus 1 Positive Control	Volume for 379 RNA Samples plus 4 Negative Controls plus 1 Positive Control
TaqPath™ 1-Step Multiplex Master Mix (No ROX™) (4X)	5.00 µL	5.50 x (*n* + *y* + 1) µL	2112.0 µL
COVID-19 Assay Multiplex Advanced	1.00 µL	1.10 x (*n* + *y* + 1) µL	422.4 µL
Total Reaction Mix volume	**6.0 µL**	—	**2534.4 µL**

5. Set up the reaction plate:
   a. Pipette 6.0 µL of the Reaction Mix prepared in step 4 into each well of a MicroAmp™ Optical 384-Well Reaction Plate with Barcode.

      Plates without a barcode can be used (see "Required materials not supplied" on page 11).

   b. Gently vortex the sealed plate containing the purified sample RNA and Negative Control from the RNA extraction procedure, then centrifuge briefly to collect liquid at the bottom of the plate.

   c. Unseal the plate containing the purified sample RNA and Negative Control from the RNA extraction procedure. Add either sample RNA, Negative Control, or Positive Control to each well of the reaction plate according to Table 8 on page 48.

      IMPORTANT! To prevent sample contamination, unseal one extraction plate at a time, then reseal it after adding the samples to the RT-PCR reaction plate.

   d. Seal the plate thoroughly with MicroAmp™ Optical Adhesive Film.

      IMPORTANT! When applying the MicroAmp™ Optical Adhesive Film, ensure that pressure is applied across the entire plate and that there is a tight seal across every individual well. Failure to do so runs the risk of an improperly sealed well, leading to potential well-to-well contamination during vortexing and evaporation during PCR.

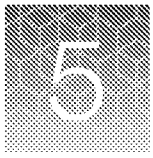
   e. Vortex the plate at the highest setting speed for 10–30 seconds with medium pressure. Move the plate around to ensure equal contact on the vortex mixer platform.

      IMPORTANT! Vortex for 10–30 seconds to ensure proper mixing. Failure to do so might result in false classification of samples.

f. Centrifuge the reaction plate for 1–2 minutes at ≥650 × *g* (≥650 RCF) to remove bubbles and to collect the liquid at the bottom of the reaction plate.

Table 8 Reaction plate volumes

Component	Volume per reaction		
	RNA Sample reaction	Positive Control reaction	Negative Control reaction
Reaction Mix	6.0 µL	6.0 µL	6.0 µL
Purified sample RNA (from RNA extraction)	14.0 µL	—	—
Positive Control (diluted TaqPath™ COVID-19 Control from step 3)	—	2.0 µL	—
Nuclease-free Water	—	12.0 µL	—
Purified Negative Control (from RNA extraction)	—	—	14.0 µL
Total volume	**20.0 µL**	**20.0 µL**	**20.0 µL**



Prepare RT-PCR reactions—COVID-19 assay plus RNase P assay (Everlywell™ COVID-19 Test Home Collection Kit)

- Protocols in this chapter 49
- Guidelines for RT-PCR 50
- Prepare the RT-PCR reactions (200-µL sample input, 96-well reaction plate, includes RNase P assay) 50
- Prepare the RT-PCR reactions (200-µL sample input, 384-well reaction plate, includes RNase P assay) 53
- Prepare the RT-PCR reactions (400-µL sample input, 96-well reaction plate, includes RNase P assay) 57
- Prepare the RT-PCR reactions (400-µL sample input, 384-well reaction plate, includes RNase P assay) 60

Protocols in this chapter

This chapter covers preparation of RT-PCR reaction plates for samples that were collected using the Everlywell™ COVID-19 Test Home Collection Kit. These RT-PCR reactions must contain both the COVID-19 Real Time PCR Assay Multiplex and the TaqMan™ SARS-CoV-2 RNase P Assay.

Note: For samples that were collected using a method other than the Everlywell™ COVID-19 Test Home Collection Kit, see Chapter 4, "Prepare RT-PCR reactions—COVID-19 assay only".

IMPORTANT! Do not combine samples collected using the Everlywell™ COVID-19 Test Home Collection Kit with samples collected via other methods on the same RT-PCR plate. The plate setup and software template files are different.

In this chapter, select the appropriate RT-PCR preparation procedure based on the following criteria:

- The original sample input volume used for RNA extraction (200 µL or 400 µL)
- The size of the RT-PCR reaction plate (96-well or a 384-well)



Guidelines for RT-PCR

IMPORTANT!

- Prepare two RT-PCR reaction plate wells for each sample and Negative Control—one for the COVID-19 assay and one for the RNase P assay—plus a single RT-PCR reaction plate well for the Positive Control.
  - 96-well reaction plates can contain up to 46 samples (2 wells each), one Negative Control (2 wells), and one Positive Control (1 well).
  - 384-well reaction plates can contain up to 189 samples (2 wells each), 2 Negative Controls (one from each extraction plate; 4 wells total), and one Positive Control (1 well).
  - For example plate layouts, see Appendix A, "Example extraction plate and RT-PCR reaction plate layouts (Everlywell™ COVID-19 Test Home Collection Kit samples)".
- Prepare the reaction plate on ice and keep it on ice until it is loaded into the real-time PCR instrument.
- Run the plate immediately after preparation. Failure to do so could result in degraded RNA samples.
- To prevent contamination, prepare reagents in a PCR workstation or equivalent amplicon-free area. Do not use the same pipette for controls and RNA samples, and always use aerosol barrier pipette tips.
- Maintain an RNase-free environment.
- Protect assays from light.
- Keep RNA samples and components on ice during use.

Prepare the RT-PCR reactions (200-µL sample input, 96-well reaction plate, includes RNase P assay)

Use this procedure under the following conditions:

- Original sample input volume of 200 µL was used for extraction
- Instrument is compatible with 96-well RT-PCR reaction plates
- Sample was collected with the Everlywell™ COVID-19 Test Home Collection Kit (RNase P assay required)

IMPORTANT! In the following procedure, each sample or Negative Control on the 96-well extraction plate is added to two wells on the 96-well reaction plate (one for the COVID-19 assay and one for the RNase P assay). For this reason, only half of the extraction plate is used per reaction plate. The extraction plate can be stored at 2–8°C for up to 48 hours.

1. If frozen, thaw the reagents on ice.
2. Gently vortex the reagents, then centrifuge briefly to collect liquid at the bottom of the tube.
3. Dilute TaqPath™ COVID-19 Control (1×10^4 copies/µL) to a working stock of 25 copies/µL:
   a. Pipet 98 µL of TaqPath™ COVID-19 Control Dilution Buffer into a microcentrifuge tube, then add 2 µL of TaqPath™ COVID-19 Control. Mix well, then centrifuge briefly.



b. Pipet 87.5 µL of TaqPath™ COVID-19 Control Dilution Buffer into a second microcentrifuge tube, then add 12.5 µL of the previous dilution. Mix well, then centrifuge briefly.

Note: The TaqPath™ COVID-19 Control does not contain the MS2 template.

4. Prepare separate reaction mixes for the COVID-19 assay and RNase P assay according to the following tables:
   a. Prepare sufficient reaction mix based on the number of RNA samples, plus one Positive Control and 2 Negative Controls per reaction plate.

All volumes include 10% overage for pipette error.

IMPORTANT! The volumes in these tables assume that you extracted sample RNA using an original sample input volume of 200 µL.

Table 9 COVID-19 Reaction Mix

Component	Volume per RNA sample or control	Volume for *n* RNA samples plus 2 controls	Volume for 46 RNA samples plus 2 controls
TaqPath™ 1-Step Multiplex Master Mix (No ROX™) (4X)	6.25 µL	6.875 x (*n* + 2) µL	330 µL
COVID-19 Real Time PCR Assay Multiplex	1.25 µL	1.375 x (*n* + 2) µL	66 µL
Nuclease-free Water	7.50 µL	8.25 x (*n* + 2) µL	396 µL
Total Reaction Mix volume	**15.0 µL**	—	**792 µL**

Table 10 RNase P Reaction Mix

Component	Volume per RNA sample or control	Volume for *n* RNA samples plus 1 Negative Control	Volume for 46 RNA samples plus 1 Negative Control
TaqPath™ 1-Step Multiplex Master Mix (No ROX™) (4X)	6.25 µL	6.875 x (*n* + 1) µL	324 µL
TaqMan™ SARS-CoV-2 RNase P Assay	1.25 µL	1.375 x (*n* + 1) µL	65 µL
Nuclease-free Water	7.50 µL	8.25 x (*n* + 1) µL	388 µL
Total Reaction Mix volume	**15.0 µL**	—	**777 µL**

5. Add each reaction mix to a reaction plate:
   a. Starting with row A, pipette 15.0 µL per well of the COVID-19 Reaction Mix into every other row of a MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL or a MicroAmp™ Optical 96-Well Reaction Plate with Barcode, 0.2 mL. See Figure 1 on page 52. Plates without a barcode can be used (see "Required materials not supplied" on page 11).

b. Pipette 15.0 µL per well of the RNase P Reaction Mix into the remaining rows of the plate.

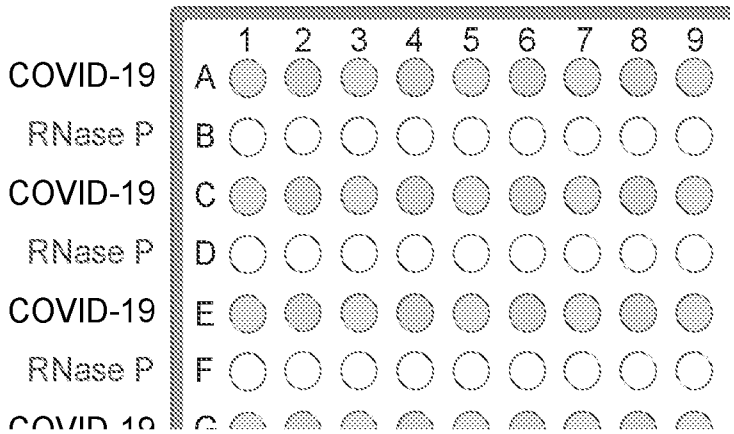


Figure 1 Assays in alternating plate rows

6. Add samples and controls to the reaction plate, using the volumes in Table 11 on page 53:
   a. Gently vortex the sealed plate containing the purified sample RNA and Negative Control from the RNA extraction procedure, then centrifuge briefly to collect liquid at the bottom of the plate.
   b. Unseal the extraction plate, then add the Negative Control from the single well of the extraction plate to wells A1 and B1 of the reaction plate, as shown in Figure 2 on page 52.
   c. Add the Positive Control (from step 3) and Nuclease-free Water to an appropriate well of the reaction plate (containing the COVID-19 assay).
      There is no RNase P well for the Positive Control.
   d. Add each RNA sample from the extraction plate to adjacent COVID-19 assay and RNase P assay wells of the reaction plate, as shown in the following figure.

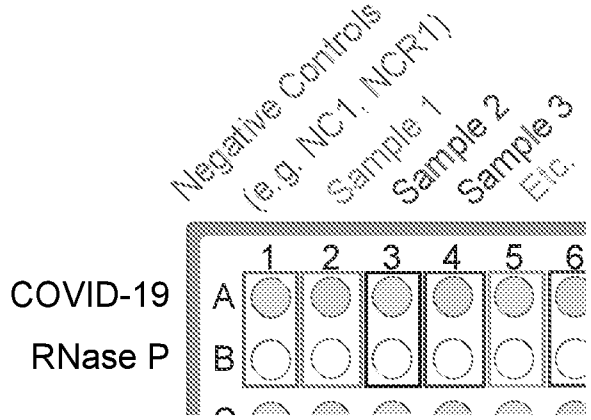


Figure 2 Paired sample and Negative Control wells for each assay

Table 11 Reaction plate volumes

Component	Volume per reaction		
	RNA Sample reaction	Positive Control reaction	Negative Control reaction
Reaction Mix (COVID-19 or RNase P)	15.0 µL	15.0 µL	15.0 µL
Purified sample RNA (from RNA extraction)	10.0 µL	—	—
Positive Control (diluted TaqPath™ COVID-19 Control, from step 3)	—	2.0 µL	—
Nuclease-free Water	—	8.0 µL	—
Purified Negative Control (from RNA extraction)	—	—	10.0 µL
Total volume	**25.0 µL**	**25.0 µL**	**25.0 µL**

7. Seal, vortex, and centrifuge the reaction plate:
   a. Seal the reaction plate thoroughly with MicroAmp™ Optical Adhesive Film.

   IMPORTANT! When applying the MicroAmp™ Optical Adhesive Film, ensure that pressure is applied across the entire plate and that there is a tight seal across every individual well. Failure to do so runs the risk of an improperly sealed well, leading to potential well-to-well contamination during vortexing and evaporation during PCR.

   b. Vortex the reaction plate at the highest setting speed for 10–30 seconds with medium pressure. Move the plate around to ensure equal contact on the vortex mixer platform.

   IMPORTANT! Vortex for 10–30 seconds to ensure proper mixing. Failure to do so might result in false classification of samples.

   c. Centrifuge the reaction plate for 1–2 minutes at ≥650 × *g* (≥650 RCF) to remove bubbles and to collect the liquid at the bottom of the reaction plate.

Prepare the RT-PCR reactions (200-µL sample input, 384-well reaction plate, includes RNase P assay)

Use this procedure under the following conditions:

- Original sample input volume of 200 µL was used for extraction
- Instrument is compatible with 384-well RT-PCR reaction plates
- Sample was collected with the Everlywell™ COVID-19 Test Home Collection Kit (RNase P assay required)

Note: You can combine multiple 96-well extraction plates on a single 384-well RT-PCR reaction plate.



Chapter 5 Prepare RT-PCR reactions—COVID-19 assay plus RNase P assay (Everlywell™ COVID-19 Test Home Collection Kit)

Prepare the RT-PCR reactions (200-µL sample input, 384-well reaction plate, includes RNase P assay)

1. If frozen, thaw the reagents on ice.

2. Gently vortex the reagents, then centrifuge briefly to collect liquid at the bottom of the tube.

3. Dilute TaqPath™ COVID-19 Control (1×10^4 copies/µL) to a working stock of 25 copies/µL:
   a. Pipet 98 µL of TaqPath™ COVID-19 Control Dilution Buffer into a microcentrifuge tube, then add 2 µL of TaqPath™ COVID-19 Control. Mix well, then centrifuge briefly.

   b. Pipet 87.5 µL of TaqPath™ COVID-19 Control Dilution Buffer into a second microcentrifuge tube, then add 12.5 µL of the previous dilution. Mix well, then centrifuge briefly.

Note: The TaqPath™ COVID-19 Control does not contain the MS2 template.

4. Prepare separate reaction mixes for the COVID-19 assay and RNase P assay according to the following tables:
   a. Prepare sufficient reaction mix based on the number of RNA samples, plus one Positive Control per 384-well reaction plate, and 2 Negative Controls from each extraction run.

   For example, if RNA samples from 2 96-well extraction runs are being combined on one 384-well reaction plate, then prepare 4 Negative Control wells on that reaction plate (2 extraction plates × 2 assay types).

All volumes include 10% overage for pipette error.

IMPORTANT! The volumes in these tables assume that you extracted sample RNA using an original sample input volume of 200 µL.

Table 12 COVID-19 Reaction Mix

Component	Volume per RNA Sample or Control	Volume for *n* RNA Samples plus *y* Negative Controls plus 1 Positive Control	Volume for 189 RNA Samples plus 2 Negative Controls plus 1 Positive Control
TaqPath™ 1-Step Multiplex Master Mix (No ROX™) (4X)	5.00 µL	5.50 x (*n* + *y* + 1) µL	1056 µL
COVID-19 Real Time PCR Assay Multiplex	1.00 µL	1.10 x (*n* + *y* + 1) µL	211 µL
Nuclease-free Water	4.00 µL	4.40 x (*n* + *y* + 1) µL	845 µL
Total Reaction Mix volume	**10.0 µL**	—	**2112 µL**

Table 13 RNase P Reaction Mix

Component	Volume per RNA Sample or control	Volume for *n* RNA Samples plus *y* Negative Controls	Volume for 189 RNA Samples plus 2 Negative Controls
TaqPath™ 1-Step Multiplex Master Mix (No ROX™) (4X)	5.00 µL	5.50 x (*n* + *y*) µL	1050.5 µL
TaqMan™ SARS-CoV-2 RNase P Assay	1.00 µL	1.10 x (*n* + *y*) µL	210.1 µL
Nuclease-free Water	4.00 µL	4.40 x (*n* + *y*) µL	840.4 µL
Total Reaction Mix volume	**10.0 µL**	—	**2101.0 µL**

5. Add each reaction mix to a reaction plate:
   a. Starting with row A, pipette 10.0 µL per well of the COVID-19 Reaction Mix into every other row of a MicroAmp™ Optical 384-Well Reaction Plate with Barcode. See Figure 3 on page 55. Plates without a barcode can be used (see "Required materials not supplied" on page 11).
   b. Pipette 10.0 µL per well of the RNase P Reaction Mix into the remaining rows of the plate.

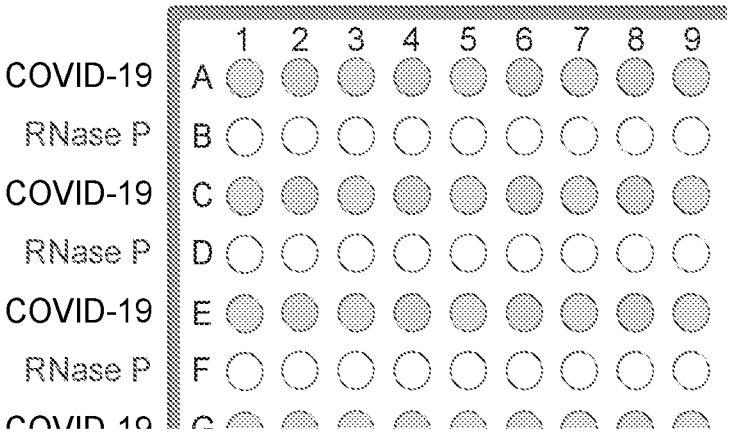


Figure 3 Assays in alternating plate rows

6. Add samples and controls to the reaction plate, using the volumes in Table 14 on page 56:
   a. Gently vortex the sealed plate containing the purified sample RNA and Negative Control from the RNA extraction procedure, then centrifuge briefly to collect liquid at the bottom of the plate.

   IMPORTANT! To prevent sample contamination, unseal one extraction plate at a time, then reseal it after adding the samples to the reaction plate.

   b. Unseal the extraction plate, then add the first Negative Control from the single well of the extraction plate to wells A1 and B1 of the reaction plate, as shown in Figure 4 on page 56.

   Note: If you combine multiple extraction plates on the reaction plate, add the Negative Control from each extraction plate to adjacent COVID-19 assay and RNase P assay wells.

c. Add the Positive Control (from step 3) and Nuclease-free Water to an appropriate well of the reaction plate (containing the COVID-19 assay).

Note:

- There is no RNase P well for the Positive Control.
- Only one Positive Control is required per 384-well plate, even if you combine multiple extraction plates.

d. Add each RNA sample from the extraction plate to adjacent COVID-19 assay and RNase P assay wells of the reaction plate, as shown in the following figure.

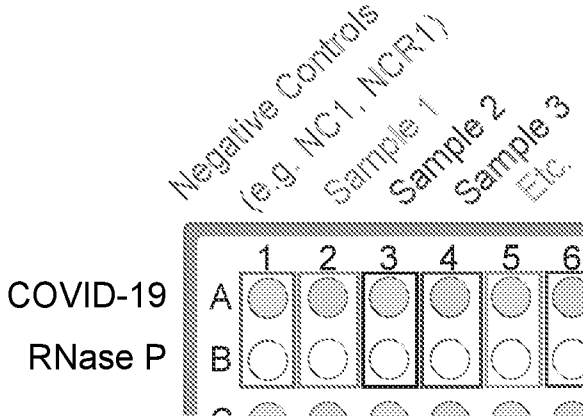


Figure 4 Paired sample and Negative Control wells for each assay

Table 14 Reaction plate volumes

Component	Volume per reaction		
	RNA Sample reaction	Positive Control reaction	Negative Control reaction
Reaction Mix (COVID-19 or RNase P)	10.0 µL	10.0 µL	10.0 µL
Purified sample RNA (from RNA extraction)	10.0 µL	—	—
Positive Control (diluted TaqPath™ COVID-19 Control from step 3)	—	2.0 µL	—
Nuclease-free Water	—	8.0 µL	—
Purified Negative Control (from RNA extraction)	—	—	10.0 µL
Total volume	**20.0 µL**	**20.0 µL**	**20.0 µL**

7. Seal, vortex, and centrifuge the reaction plate:
   a. Seal the reaction plate thoroughly with MicroAmp™ Optical Adhesive Film.

   IMPORTANT! When applying the MicroAmp™ Optical Adhesive Film, ensure that pressure is applied across the entire plate and that there is a tight seal across every individual well. Failure to do so runs the risk of an improperly sealed well, leading to potential well-to-well contamination during vortexing and evaporation during PCR.

   b. Vortex the reaction plate at the highest setting speed for 10–30 seconds with medium pressure. Move the plate around to ensure equal contact on the vortex mixer platform.

   IMPORTANT! Vortex for 10–30 seconds to ensure proper mixing. Failure to do so might result in false classification of samples.

   c. Centrifuge the reaction plate for 1–2 minutes at ≥650 × *g* (≥650 RCF) to remove bubbles and to collect the liquid at the bottom of the reaction plate.

Prepare the RT-PCR reactions (400-µL sample input, 96-well reaction plate, includes RNase P assay)

Use this procedure under the following conditions:

- Original sample input volume of 400 µL was used for extraction
- Instrument is compatible with 96-well RT-PCR reaction plates
- Sample was collected with the Everlywell™ COVID-19 Test Home Collection Kit (RNase P assay required)

IMPORTANT! In the following procedure, each sample or Negative Control on the 96-well extraction plate is added to two wells on the 96-well reaction plate (one for the COVID-19 assay and one for the RNase P assay). For this reason, only half of the extraction plate is used per reaction plate. The extraction plate can be stored at 2–8°C for up to 48 hours.

1. If frozen, thaw the reagents on ice.
2. Gently vortex the reagents, then centrifuge briefly to collect liquid at the bottom of the tube.
3. Dilute TaqPath™ COVID-19 Control (1×10^4 copies/µL) to a working stock of 25 copies/µL:
   a. Pipet 98 µL of TaqPath™ COVID-19 Control Dilution Buffer into a microcentrifuge tube, then add 2 µL of TaqPath™ COVID-19 Control. Mix well, then centrifuge briefly.
   b. Pipet 87.5 µL of TaqPath™ COVID-19 Control Dilution Buffer into a second microcentrifuge tube, then add 12.5 µL of the previous dilution. Mix well, then centrifuge briefly.

Note: The TaqPath™ COVID-19 Control does not contain the MS2 template.

4. Prepare separate reaction mixes for the COVID-19 assay and RNase P assay according to the following tables:
   a. Prepare sufficient reaction mix based on the number of RNA samples, plus one Positive Control and 2 Negative Controls per reaction plate.

All volumes include 10% overage for pipette error.

IMPORTANT! The volumes in these tables assume that you extracted sample RNA using an original sample input volume of 400 µL.

Table 15 COVID-19 Reaction Mix

Component	Volume per RNA Sample or control	Volume for *n* RNA Samples plus 2 controls	Volume for 46 RNA samples plus 2 controls
TaqPath™ 1-Step Multiplex Master Mix (No ROX™) (4X)	6.25 µL	6.875 x (*n* + 2) µL	330 µL
COVID-19 Real Time PCR Assay Multiplex	1.25 µL	1.375 x (*n* + 2) µL	66 µL
Nuclease-free Water	12.50 µL	13.75 x (*n* + 2) µL	660 µL
Total Reaction Mix volume	**20.0 µL**	—	**1056 µL**

Table 16 RNase P Reaction Mix

Component	Volume per RNA sample or control	Volume for *n* RNA samples plus 1 Negative Control	Volume for 46 RNA samples plus 1 Negative Control
TaqPath™ 1-Step Multiplex Master Mix (No ROX™) (4X)	6.25 µL	6.875 x (*n* + 1) µL	323.1 µL
TaqMan™ SARS-CoV-2 RNase P Assay	1.25 µL	1.375 x (*n* + 1) µL	64.6 µL
Nuclease-free Water	12.50 µL	13.75 x (*n* + 1) µL	646.3 µL
Total Reaction Mix volume	**20.0 µL**	—	**1034.0 µL**

5. Add each reaction mix to a reaction plate:
   a. Starting with row A, pipette 20.0 µL per well of the COVID-19 Reaction Mix into every other row of a MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL or a MicroAmp™ Optical 96-Well Reaction Plate with Barcode, 0.2 mL. See Figure 5 on page 59.

   Plates without a barcode can be used (see "Required materials not supplied" on page 11).

b. Pipette 20.0 µL per well of the RNase P Reaction Mix into the remaining rows of the plate.

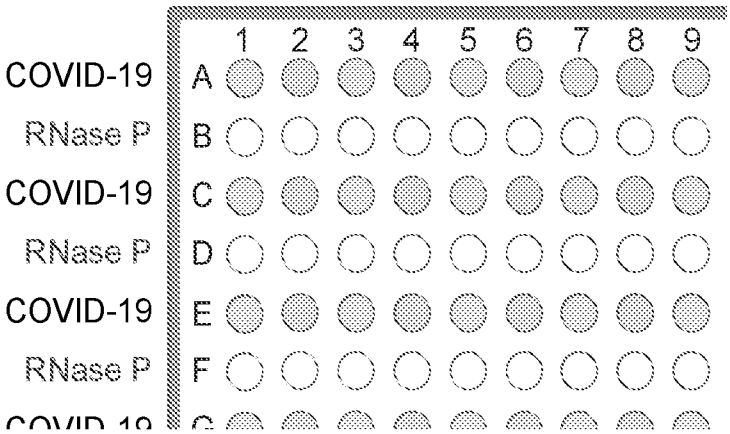


Figure 5 Assays in alternating plate rows

6. Add samples and controls to the reaction plate, using the volumes in Table 17 on page 60:
   a. Gently vortex the sealed plate containing the purified sample RNA and Negative Control from the RNA extraction procedure, then centrifuge briefly to collect liquid at the bottom of the plate.
   b. Unseal the extraction plate, then add Negative Control from the single well of the extraction plate to wells A1 and B1 of the reaction plate, as shown in Figure 6 on page 59.
   c. Add the Positive Control (from step 3) and Nuclease-free Water to an appropriate well of the reaction plate (containing the COVID-19 assay).
      There is no RNase P well for the Positive Control.
   d. Add each RNA sample from the extraction plate to adjacent COVID-19 assay and RNase P assay wells of the reaction plate, as shown in the following figure.

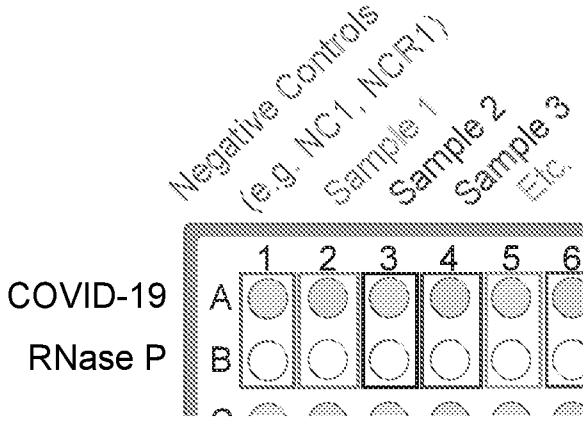


Figure 6 Paired sample and Negative Control wells for each assay

Table 17 Reaction plate volumes

Component	Volume per reaction		
	RNA Sample reaction	Positive Control reaction	Negative Control reaction
Reaction Mix (COVID-19 or RNase P)	20.0 µL	20.0 µL	20.0 µL
Purified sample RNA (from RNA extraction)	5.0 µL	—	—
Positive Control (diluted TaqPath™ COVID-19 Control from step 3)	—	2.0 µL	—
Nuclease-free Water	—	3.0 µL	—
Purified Negative Control (from RNA extraction)	—	—	5.0 µL
Total volume	**25.0 µL**	**25.0 µL**	**25.0 µL**

7. Seal, vortex, and centrifuge the reaction plate:
   a. Seal the reaction plate thoroughly with MicroAmp™ Optical Adhesive Film.

      IMPORTANT! When applying the MicroAmp™ Optical Adhesive Film, ensure that pressure is applied across the entire plate and that there is a tight seal across every individual well. Failure to do so runs the risk of an improperly sealed well, leading to potential well-to-well contamination during vortexing and evaporation during PCR.

   b. Vortex the reaction plate at the highest setting speed for 10–30 seconds with medium pressure. Move the plate around to ensure equal contact on the vortex mixer platform.

      IMPORTANT! Vortex for 10–30 seconds to ensure proper mixing. Failure to do so might result in false classification of samples.

   c. Centrifuge the reaction plate for 1–2 minutes at ≥650 × *g* (≥650 RCF) to remove bubbles and to collect the liquid at the bottom of the reaction plate.

Prepare the RT-PCR reactions (400-µL sample input, 384-well reaction plate, includes RNase P assay)

Use this procedure under the following conditions:

- Original sample input volume of 400 µL was used for extraction
- Instrument is compatible with 384-well RT-PCR reaction plates
- Sample was collected with the Everlywell™ COVID-19 Test Home Collection Kit (RNase P assay required)

Note: You can combine multiple 96-well extraction plates on a single 384-well RT-PCR reaction plate.

1. If frozen, thaw the reagents on ice.
2. Gently vortex the reagents, then centrifuge briefly to collect liquid at the bottom of the tube.
3. Dilute TaqPath™ COVID-19 Control (1×10^4 copies/µL) to a working stock of 25 copies/µL:
   a. Pipet 98 µL of TaqPath™ COVID-19 Control Dilution Buffer into a microcentrifuge tube, then add 2 µL of TaqPath™ COVID-19 Control. Mix well, then centrifuge briefly.
   b. Pipet 87.5 µL of TaqPath™ COVID-19 Control Dilution Buffer into a second microcentrifuge tube, then add 12.5 µL of the previous dilution. Mix well, then centrifuge briefly.

Note: The TaqPath™ COVID-19 Control does not contain the MS2 template.

4. Prepare separate reaction mixes for the COVID-19 assay and RNase P assay according to the following tables:
   a. Prepare sufficient reaction mix based on the number of RNA samples, plus one Positive Control per 384-well reaction plate, and 2 Negative Controls from each extraction run.

      For example, if RNA samples from 2 96-well extraction runs are being combined on one 384-well reaction plate, then prepare 4 Negative Control wells on that reaction plate (2 extraction plates × 2 assay types).

All volumes include 10% overage for pipette error.

IMPORTANT! The volumes in these tables assume that you extracted sample RNA using an original sample input volume of 400 µL.

Table 18 COVID-19 Reaction Mix

Component	Volume per RNA Sample or control	Volume for *n* RNA Samples plus *y* Negative Controls plus 1 Positive Control	Volume for 189 RNA Samples plus 2 Negative Controls plus 1 Positive Control
TaqPath™ 1-Step Multiplex Master Mix (No ROX™) (4X)	5.00 µL	5.50 x (*n* + *y* + 1) µL	1056 µL
COVID-19 Real Time PCR Assay Multiplex	1.00 µL	1.10 x (*n* + *y* + 1) µL	211 µL
Nuclease-free Water	9.00 µL	9.90 x (*n* + *y* + 1) µL	1901 µL
Total Reaction Mix volume	**15.0 µL**	—	**3168 µL**

Table 19 RNase P Reaction Mix

Component	Volume per RNA Sample or control	Volume for *n* RNA Samples plus *y* Negative Controls	Volume for 189 RNA Samples plus 2 Negative Controls
TaqPath™ 1-Step Multiplex Master Mix (No ROX™) (4X)	5.00 µL	5.50 x (*n* + *y*) µL	1050.5 µL
TaqMan™ SARS-CoV-2 RNase P Assay	1.00 µL	1.10 x (*n* + *y*) µL	210.1 µL
Nuclease-free Water	9.00 µL	9.90 x (*n* + *y* + 1) µL	1890.9 µL
Total Reaction Mix volume	**15.0 µL**	—	**3151.5 µL**

5. Add each reaction mix to a reaction plate:
   a. Starting with row A, pipette 15.0 µL per well of the COVID-19 Reaction Mix into every other row of a MicroAmp™ Optical 384-Well Reaction Plate with Barcode. See Figure 7 on page 62. Plates without a barcode can be used (see "Required materials not supplied" on page 11).
   b. Pipette 15.0 µL per well of the RNase P Reaction Mix into the remaining rows of the plate.

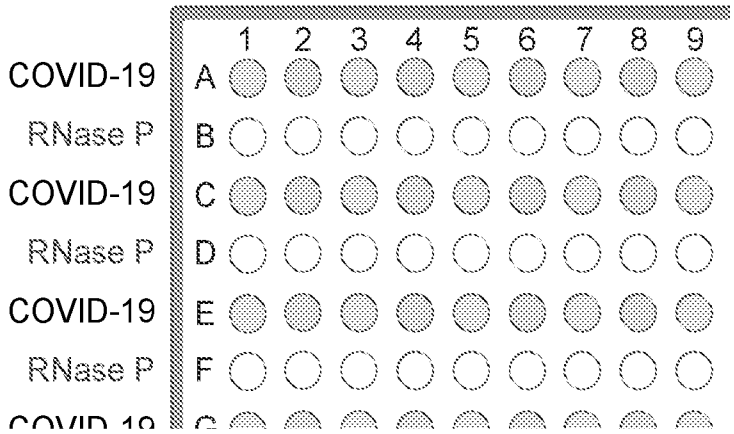


Figure 7 Assays in alternating plate rows

6. Add samples and controls to the reaction plate, using the volumes in Table 20 on page 63:
   a. Gently vortex the sealed plate containing the purified sample RNA and Negative Control from the RNA extraction procedure, then centrifuge briefly to collect liquid at the bottom of the plate.

   IMPORTANT! To prevent sample contamination, unseal one extraction plate at a time, then reseal it after adding the samples to the reaction plate.

   b. Unseal the extraction plate, then add the Negative Control from the single well of the extraction plate to wells A1 and B1 of the reaction plate, as shown in Figure 8 on page 63.

   Note: If you combine multiple extraction plates on the reaction plate, add the Negative Control from each extraction plate to adjacent COVID-19 assay and RNase P assay wells.

c. Add the Positive Control (from step 3) and Nuclease-free Water to an appropriate well of the reaction plate (containing the COVID-19 assay).

Note:

- There is no RNase P well for the Positive Control.
- Only one Positive Control is required per 384-well plate, even if you combine multiple extraction plates.

d. Add each RNA sample from the extraction plate to adjacent COVID-19 assay and RNase P assay wells of the reaction plate, as shown in the following figure.

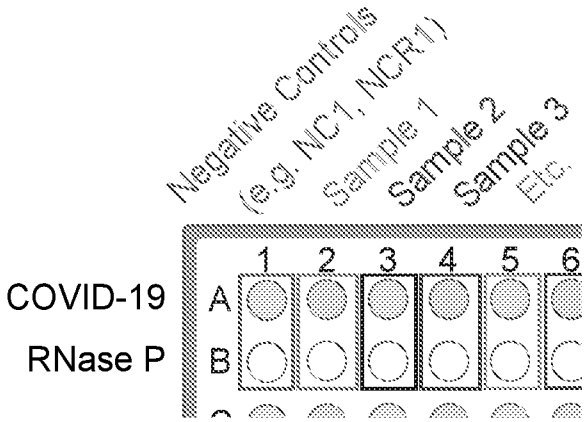


Figure 8 Paired sample and Negative Control wells for each assay

Table 20 Reaction plate volumes

Component	Volume per reaction		
	RNA Sample reaction	Positive Control reaction	Negative Control reaction
Reaction Mix (COVID-19 or RNase P)	15.0 µL	15.0 µL	15.0 µL
Purified sample RNA (from RNA extraction)	5.0 µL	—	—
Positive Control (diluted TaqPath™ COVID-19 Control from step 3)	—	2.0 µL	—
Nuclease-free Water	—	3.0 µL	—
Purified Negative Control (from RNA extraction)	—	—	5.0 µL
Total volume	**20.0 µL**	**20.0 µL**	**20.0 µL**



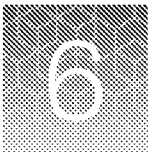
7. Seal, vortex, and centrifuge the reaction plate:
   a. Seal the reaction plate thoroughly with MicroAmp™ Optical Adhesive Film.

   IMPORTANT! When applying the MicroAmp™ Optical Adhesive Film, ensure that pressure is applied across the entire plate and that there is a tight seal across every individual well. Failure to do so runs the risk of an improperly sealed well, leading to potential well-to-well contamination during vortexing and evaporation during PCR.

   b. Vortex the reaction plate at the highest setting speed for 10–30 seconds with medium pressure. Move the plate around to ensure equal contact on the vortex mixer platform.

   IMPORTANT! Vortex for 10–30 seconds to ensure proper mixing. Failure to do so might result in false classification of samples.

   c. Centrifuge the reaction plate for 1–2 minutes at ≥650 × *g* (≥650 RCF) to remove bubbles and to collect the liquid at the bottom of the reaction plate.



Perform RT-PCR using the Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument

- Dye calibration for the 7500 Real-Time PCR Instrument series 65
- Transfer the template (SDT) file for the 7500 Fast Dx Real-Time PCR Instrument 65
- Set up and run the 7500 Fast Dx Real-Time PCR Instrument—COVID-19 assay only 66
- Set up and run the 7500 Fast Dx Real-Time PCR Instrument—COVID-19 plus RNase P assay .. 68

Dye calibration for the 7500 Real-Time PCR Instrument series

A maintained instrument will be calibrated for many dyes. In addition to those dyes, the instrument operator must calibrate the instrument for ABY™ dye and JUN™ dye that are used with this kit. For all other assays, refer to the standard calibration process.

Transfer the template (SDT) file for the 7500 Fast Dx Real-Time PCR Instrument

The template (SDT) file contains the settings for the instrument run. It is installed on the computer with Applied Biosystems™ COVID-19 Interpretive Software, and must be transferred via a USB drive or other method to the computer on which SDS Software v1.4.1 is installed.

IMPORTANT! Be careful to select the appropriate template file for your instrument, software version, and assay type. Failure to do so can cause errors in the analysis.

---

1. On the computer with Applied Biosystems™ COVID-19 Interpretive Software, navigate to one of the following directories, based on whether the RT-PCR reaction contains only the COVID-19 assay, or both the COVID-19 assay and the RNase P assay (for Everlywell™ COVID-19 Test Home Collection Kit samples):

   - `<...>\Applied Biosystems\COVID-19 Interpretive Software\Client\docs\User Documents\COVID-19`
   - `<...>\Applied Biosystems\COVID-19 Interpretive Software\Client\docs\User Documents\COVID-19 + RNase P`

   <...> is the installation directory

2. Select the correct SDT file, based on whether the RT-PCR reaction contains only the COVID-19 assay, or both the COVID-19 assay and the RNase P assay (for Everlywell™ COVID-19 Test Home Collection Kit samples):

Assay type	Template name
COVID-19 only	`TaqPath COVID-19 Kit Template 7500fastDx sds1_4_1 v1-4.sdt`
COVID-19 and RNase P	`TaqPath COVID-19 and RNaseP Template 7500fastDx sds1_4_1 v1-0.sdt`

3. Transfer the SDT file to the computer with SDS Software v1.4.1, using a USB drive or other method.

Set up and run the 7500 Fast Dx Real-Time PCR Instrument—COVID-19 assay only

This section covers instrument set-up and operation for reaction plates that contain only the COVID-19 assay.

For plates that contain both the COVID-19 assay and RNase P assay, see "Set up and run the 7500 Fast Dx Real-Time PCR Instrument—COVID-19 plus RNase P assay" on page 68.

For more information about the instrument, see the documents listed in "Related documentation" on page 122.

1. Using SDS Software v1.4.1, open the SDT file that you transferred in "Transfer the template (SDT) file for the 7500 Fast Dx Real-Time PCR Instrument" on page 65.

   IMPORTANT! Be careful to select the appropriate template file for your instrument, software version, and assay type. Failure to do so can cause errors in the analysis.

2. Confirm the run settings in the template and adjust as necessary.

   - **Assay**: **Standard Curve (Absolute Quantitation)**
   - **Run mode**: **Standard 7500**
   - **Passive reference**: **None**
   - **Sample volume**: **25 µL**

   IMPORTANT! The passive reference must be set to **None**.

3. Confirm that the reporter dye and the detector pairs are correct in the **Detector Manager** in the **Tools** menu.

Reporter dye	Detector
FAM	ORF1ab
VIC	N gene
ABY	S gene
JUN	MS2

4. Confirm that the targets above are assigned to each well in the plate layout.

5. Confirm the labeling of the control wells.
   - The template has one positive control and one negative control assigned to wells for reference.
   - Move the control well assignments by copying the existing control wells and pasting them according to their location on the physical plate.

6. For wells with a positive control, confirm that **Task** is set to **Standard**.

7. For wells with a negative control, confirm that **Task** is set to **NTC**.

8. Edit the plate layout to assign a unique sample name to each well in the physical plate.
   For wells with a patient sample, confirm that **Task** is set to **Unknown** for all detectors.

   Note: Wells that do not have a sample name will not be analyzed by the software.

9. Confirm the thermal protocol.

Step	Temperature	Time	Number of cycles
UNG incubation	25°C	2 minutes	1
Reverse transcription	53°C	10 minutes	1
Activation	95°C	2 minutes	1
Denaturation	95°C	3 seconds	40
Anneal / extension	60°C	30 seconds	

10. Click **Save As**, enter a file name, then click **Save**.

11. Reopen the file to connect the computer to the instrument, load the plate, then start the run on the real-time PCR instrument.

12. After the instrument run is complete, open the SDS file in SDS Software v1.4.1. Analyze, then save the file.

Set up and run the 7500 Fast Dx Real-Time PCR Instrument —COVID-19 plus RNase P assay

This section covers instrument set-up and operation for reaction plates that contain both the COVID-19 assay and the RNase P assay. Both assays are required for samples collected using the Everlywell™ COVID-19 Test Home Collection Kit.

For plates that contain only the COVID-19 assay, see "Set up and run the 7500 Fast Dx Real-Time PCR Instrument—COVID-19 assay only" on page 66.

For more information about the instrument, see the documents listed in "Related documentation" on page 122.

1. Using SDS Software v1.4.1, open the SDT file that you transferred in "Transfer the template (SDT) file for the 7500 Fast Dx Real-Time PCR Instrument" on page 65.

   IMPORTANT! Be careful to select the appropriate template file for your instrument, software version, and assay type. Failure to do so can cause errors in the analysis.

2. Confirm the run settings in the template and adjust as necessary.
   - **Assay: Standard Curve (Absolute Quantitation)**
   - **Run mode: Standard 7500**
   - **Passive reference: None**
   - **Sample volume: 25 µL**

   IMPORTANT! The passive reference must be set to **None**.

3. Confirm that the reporter dye and the detector pairs are correct in the **Detector Manager** in the **Tools** menu.

Reporter dye	Detector
COVID-19 assay	
FAM	ORF1ab
VIC	N gene
ABY	S gene
JUN	MS2
RNase P assay	
VIC	RNaseP

4. Confirm that the detectors above are assigned to the appropriate wells in the plate layout.

   Note:
   - Wells containing the RNase P assay have only the RNaseP detector assigned.
   - Wells containing the COVID-19 assay have all other detectors assigned.

5. Confirm the labeling of the control wells.
   - The template has one positive control and 2 negative controls (one each for the COVID-19 assay and RNase P assay) assigned to wells.
   - Move the control well assignments by copying the existing control wells and pasting them according to their location on the physical plate.
6. For wells with a positive control, confirm that **Task** is set to **Standard**.
7. For wells with a negative control, confirm that **Task** is set to **NTC**.
8. Edit the plate layout to assign the same sample name to each paired COVID-19 assay well and RNase P assay well in the physical plate.

   IMPORTANT! Paired sample assay wells that do not have the same sample name will not be analyzed by the software.

   For wells with a patient sample, confirm that **Task** is set to **Unknown** for all detectors.
9. Confirm the thermal protocol.

Step	Temperature	Time	Number of cycles
UNG incubation	25°C	2 minutes	1
Reverse transcription	53°C	10 minutes	1
Activation	95°C	2 minutes	1
Denaturation	95°C	3 seconds	40
Anneal / extension	60°C	30 seconds	

10. Click **Save As**, enter a file name, then click **Save**.
11. Reopen the file to connect the computer to the instrument, load the plate, then start the run on the instrument.
12. After the instrument run is complete, open the SDS file in SDS Software v1.4.1. Analyze, then save the file.



Perform RT-PCR using the Applied Biosystems™ 7500 Fast Real-Time PCR Instrument

- Dye calibration for the 7500 Real-Time PCR Instrument series 70
- Transfer the template (SDT or EDT) file for the 7500 Fast Real-Time PCR Instrument 71
- Set up and run the 7500 Fast Real-Time PCR Instrument (SDS Software v1.5.1)—COVID-19 assay only 72
- Set up and run the 7500 Fast Real-Time PCR Instrument (7500 Software v2.3)—COVID-19 assay only 74
- Set up and run the 7500 Fast Real-Time PCR Instrument (SDS Software v1.5.1)—COVID-19 plus RNase P assay 75
- Set up and run the 7500 Fast Real-Time PCR Instrument (7500 Software v2.3)—COVID-19 plus RNase P assay 77

Dye calibration for the 7500 Real-Time PCR Instrument series

A maintained instrument will be calibrated for many dyes. In addition to those dyes, the instrument operator must calibrate the instrument for ABY™ dye and JUN™ dye that are used with this kit. For all other assays, refer to the standard calibration process.

Transfer the template (SDT or EDT) file for the 7500 Fast Real-Time PCR Instrument

The template (SDT or EDT) file contains the settings for the instrument run. It is installed on the computer with Applied Biosystems™ COVID-19 Interpretive Software, and must be transferred via a USB drive or other method to the computer on which instrument data collection software is installed.

IMPORTANT! Be careful to select the appropriate template file for your instrument, software version, and assay type. Failure to do so can cause errors in the analysis.

1. On the computer with Applied Biosystems™ COVID-19 Interpretive Software, navigate to one of the following directories, based on whether the RT-PCR reaction contains only the COVID-19 assay, or both the COVID-19 assay and the RNase P assay (for Everlywell™ COVID-19 Test Home Collection Kit samples):
   - `<...>\Applied Biosystems\COVID-19 Interpretive Software\Client\docs\User Documents\COVID-19`
   - `<...>\Applied Biosystems\COVID-19 Interpretive Software\Client\docs\User Documents\COVID-19 + RNase P`

   <...> is the installation directory
2. Select the correct template file, based on whether the RT-PCR reaction contains only the COVID-19 assay, or both the COVID-19 assay and RNase P assay (for Everlywell™ COVID-19 Test Home Collection Kit samples):

Table 21 COVID-19 assay only

Data collection software version	Template file
SDS Software v1.5.1	`TaqPath COVID-19 Kit Template 7500fast sds1_5_1 v1-4.sdt`
7500 Software v2.3	`TaqPath COVID-19 Kit Template 7500fast sds2_3 v1-2.edt`

Table 22 COVID-19 assay and RNase P assay

Data collection software version	Template file
SDS Software v1.5.1	`TaqPath COVID-19 and RNaseP Template 7500fast sds1_5_1 v1-0.sdt`
7500 Software v2.3	`TaqPath COVID-19 and RNaseP Template 7500fast sds2_3 v1-0.edt`

3. Select the correct SDT or EDT file for the version of the instrument software that you are using:

Data collection software version	Template file
SDS Software v1.5.1	`TaqPath COVID-19 Kit Template 7500fast sds1_5_1 v1-4.sdt`
7500 Software v2.3	`TaqPath COVID-19 Kit Template 7500fast sds2_3 v1-2.edt`

4. Transfer the appropriate SDT or EDT file to the computer with your data collection software, using a USB drive or other method.

Set up and run the 7500 Fast Real-Time PCR Instrument (SDS Software v1.5.1)—COVID-19 assay only

This section covers instrument set-up and operation for reaction plates that contain only the COVID-19 assay, and is specific for the 7500 Fast Real-Time PCR Instrument using SDS Software v1.5.1.

For plates that contain both the COVID-19 assay and RNase P assay, see "Set up and run the 7500 Fast Real-Time PCR Instrument (SDS Software v1.5.1)—COVID-19 plus RNase P assay" on page 75.

For more information about the instrument, see the documents listed in "Related documentation" on page 122.

1. Using SDS Software v1.5.1, access the appropriate template file.
   a. Create a new experiment.
   b. In the **Template** field, browse to, then open the SDT file that you transferred in "Transfer the template (SDT or EDT) file for the 7500 Fast Real-Time PCR Instrument" on page 71.

IMPORTANT! Be careful to select the appropriate template file for your instrument, software version, and assay type. Failure to do so can cause errors in the analysis.

2. Confirm the run settings in the template and adjust as necessary.
   - **Assay**: **Standard Curve (Absolute Quantitation)**
   - **Run mode**: **Standard 7500**
   - **Passive reference**: **None**
   - **Sample volume**: **25 µL**

IMPORTANT! The passive reference must be set to **None**.

3. Confirm that the reporter dye and the detector pairs are correct in the **Detector Manager** in the **Tools** menu.

Reporter dye	Detector
FAM	ORF1ab
VIC	N gene
ABY	S gene
JUN	MS2

4. Confirm that the targets above are assigned to each well in the plate layout.

5. Confirm the labeling of the control wells.
   - The template has one positive control (PC) and one negative control (NC) assigned to wells for reference.
   - Move the control well assignments by copying the existing control wells and pasting them according to their location on the physical plate.

6. For wells with a positive control, confirm that **Task** is set to **Standard**.

7. For wells with a negative control, confirm that **Task** is set to **NTC**.

8. Edit the plate layout to assign a unique sample name to each well in the physical plate.
   For wells with a patient sample, ensure that **Task** is set to **Unknown** for all detectors.

Note: Wells that do not have a sample name will not be analyzed by the software.

9. Confirm the thermal protocol.

Step	Temperature	Time	Number of cycles
UNG incubation	25°C	2 minutes	1
Reverse transcription	53°C	10 minutes	1
Activation	95°C	2 minutes	1
Denaturation	95°C	3 seconds	40
Anneal / extension	60°C	30 seconds	

10. Click **Save As**, enter a file name, then click **Save**.

11. Reopen the file, load the plate, then start the run on the instrument.

12. After the instrument run is complete, open the SDS file in SDS Software v1.5.1. Analyze, then save the file.

Set up and run the 7500 Fast Real-Time PCR Instrument (7500 Software v2.3)—COVID-19 assay only

This section covers instrument set-up and operation for reaction plates that contain only the COVID-19 assay, and is specific for the 7500 Fast Real-Time PCR Instrument using SDS Software v2.3.

For plates that contain both the COVID-19 assay and RNase P assay, see "Set up and run the 7500 Fast Real-Time PCR Instrument (7500 Software v2.3)—COVID-19 plus RNase P assay" on page 77.

For more information about the instrument, see the documents listed in "Related documentation" on page 122.

1. In the 7500 Software v2.3 home page, click **Template**.
2. Browse to, then open the EDT file that you transferred in "Transfer the template (SDT or EDT) file for the 7500 Fast Real-Time PCR Instrument" on page 71.

   IMPORTANT! Be careful to select the appropriate template file for your instrument, software version, and assay type. Failure to do so can cause errors in the analysis.
3. In the **Experiment Properties** window, enter or confirm the following information:
   - **Experiment name**: Enter a unique name
   - **Instrument type**: **7500 Fast (96 wells)**
   - **Type of experiment**: **Quantitation - Standard Curve**
   - **Reagents**: **TaqMan™**
   - **Ramp Speed**: **Standard**
4. In the **Plate Setup** window, in the **Define Targets and Samples** tab and the **Define Targets** pane, confirm that the targets, reporter dyes, and quenchers are listed correctly.

Target	Reporter dye	Quencher
ORF1ab	FAM	None
N gene	VIC	None
S gene	ABY	None
MS2	JUN	None

5. In the **Plate Setup** window, in the **Define Targets and Samples** tab and the **Define Samples** pane, confirm the labeling of the control wells.
   - The template has one positive control (PC) and one negative control (NC) assigned to wells for reference.
   - Move the control well assignments by copying the existing control wells and pasting them according to their location on the physical plate.
   - To include additional controls, select **Add New Sample**.
6. Select **Add New Sample** to assign a unique sample name to each well in the physical plate.



7. In the **Plate Setup** window, in the **Assign Targets and Samples** tab, confirm that four targets are assigned to each well in the plate layout. To designate a target or sample to a well, select the well, then check the **Assign** checkbox.

   Note: Wells that do not have a sample name will not be analyzed by the software.

8. For wells with a positive control, confirm that **Task** is set to **S** for Standard.
9. For wells with a negative control, confirm that **Task** is set to **N** for Negative.
10. For wells with a patient sample, confirm that **Task** is set to **U** for Unknown.
11. Confirm that the **Passive Reference** is set to **None**.
12. In the **Run Method** window, confirm that the **Reaction Volume Per Well** is 25 µL, then confirm the thermal protocol.

Step	Temperature	Time	Number of cycles
UNG incubation	25°C	2 minutes	1
Reverse transcription	53°C	10 minutes	1
Activation	95°C	2 minutes	1
Denaturation	95°C	3 seconds	40
Anneal / extension	60°C	30 seconds	

13. Select **Start Run**, enter a file name, then click **Save**.
14. After the instrument run is complete, click **Analyze**, then save the file.

Set up and run the 7500 Fast Real-Time PCR Instrument (SDS Software v1.5.1)—COVID-19 plus RNase P assay

This section covers instrument set-up and operation for reaction plates that contain both the COVID-19 assay and the RNase P assay, and is specific for the 7500 Fast Real-Time PCR Instrument using SDS Software v1.5.1.

Both assays are required for samples collected using the Everlywell™ COVID-19 Test Home Collection Kit.

For plates that contain only the COVID-19 assay, see "Set up and run the 7500 Fast Real-Time PCR Instrument (SDS Software v1.5.1)—COVID-19 assay only" on page 72.

For more information about the instrument, see the documents listed in "Related documentation" on page 122.

1. Using SDS Software v1.5.1, access the appropriate template file.
   a. Create a new experiment.

b. In the **Template** field, browse to, then open the SDT file that you transferred in "Transfer the template (SDT or EDT) file for the 7500 Fast Real-Time PCR Instrument" on page 71.

IMPORTANT! Be careful to select the appropriate template file for your instrument, software version, and assay type. Failure to do so can cause errors in the analysis.

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2. Confirm the run settings in the template and adjust as necessary.
   - **Assay: Standard Curve (Absolute Quantitation)**
   - **Run mode: Standard 7500**
   - **Passive reference: None**
   - **Sample volume: 25 µL**

IMPORTANT! The passive reference must be set to **None**.

---

3. Confirm that the reporter dye and the detector pairs are correct in the **Detector Manager** in the **Tools** menu.

Reporter dye	Detector
COVID-19 assay	
FAM	ORF1ab
VIC	N gene
ABY	S gene
JUN	MS2
RNase P assay	
VIC	RNaseP

4. Confirm that the dectors above are assigned to each well in the plate layout.

Note:
- Wells containing the RNase P assay have only the RNaseP detector assigned.
- Wells containing the COVID-19 assay have all other detectors assigned.

---

5. Confirm the labeling of the control wells.
   - The template has one positive control and 2 negative controls (one each for the COVID-19 assay and RNase P assay) assigned to wells for reference.
   - Move the control well assignments by copying the existing control wells and pasting them according to their location on the physical plate.
6. For wells with a positive control, confirm that **Task** is set to **Standard**.
7. For wells with a negative control, confirm that **Task** is set to **NTC**.

8. Edit the plate layout to assign the same sample name to each paired COVID-19 assay well and RNase P assay well in the physical plate.

   IMPORTANT! Paired sample assay wells that do not have the same sample name will not be analyzed by the software.

   For wells with a patient sample, ensure that **Task** is set to **Unknown** for all detectors.

9. Confirm the thermal protocol.

Step	Temperature	Time	Number of cycles
UNG incubation	25°C	2 minutes	1
Reverse transcription	53°C	10 minutes	1
Activation	95°C	2 minutes	1
Denaturation	95°C	3 seconds	40
Anneal / extension	60°C	30 seconds	

10. Click **Save As**, enter a file name, then click **Save**.

11. Reopen the file, load the plate, then start the run on the instrument.

12. After the instrument run is complete, open the SDS file in SDS Software v1.5.1. Analyze, then save the file.

Set up and run the 7500 Fast Real-Time PCR Instrument (7500 Software v2.3)—COVID-19 plus RNase P assay

This section covers instrument set-up and operation for reaction plates that contain both the COVID-19 assay and the RNase P assay, and is specific for the 7500 Fast Real-Time PCR Instrument using SDS Software v2.3.

Both assays are required for samples collected using the Everlywell™ COVID-19 Test Home Collection Kit.

For plates that contain only the COVID-19 assay, see "Set up and run the 7500 Fast Real-Time PCR Instrument (7500 Software v2.3)—COVID-19 assay only" on page 74.

For more information about the instrument, see the documents listed in "Related documentation" on page 122.

1. In the 7500 Software v2.3 home page, click **Template**.

2. Browse to, then open the EDT file that you transferred in "Transfer the template (SDT or EDT) file for the 7500 Fast Real-Time PCR Instrument" on page 71.

   IMPORTANT! Be careful to select the appropriate template file for your instrument, software version, and assay type. Failure to do so can cause errors in the analysis.

3. In the **Experiment Properties** window, enter or confirm the following information:
   - **Experiment name**: Enter a unique name
   - **Instrument type**: **7500 Fast (96 wells)**
   - **Type of experiment**: **Quantitation - Standard Curve**
   - **Reagents**: **TaqMan™**
   - **Ramp Speed**: **Standard**

4. In the **Plate Setup** window, in the **Define Targets and Samples** tab and the **Define Targets** pane, confirm that the targets, reporter dyes, and quenchers are listed correctly.

Target	Reporter dye	Quencher
COVID-19 assay targets		
ORF1ab	FAM	None
N gene	VIC	None
S gene	ABY	None
MS2	JUN	None
RNase P assay target		
RNaseP	VIC	None

5. In the **Plate Setup** window, in the **Define Targets and Samples** tab and the **Define Samples** pane, confirm the labeling of the control wells.
   - The template has one positive control and 2 negative controls (one each for the COVID-19 assay and RNase P assay) assigned to wells for reference.
   - Move the control well assignments by copying the existing control wells and pasting them according to their location on the physical plate.
   - To include additional controls, select **Add New Sample**.

6. Select **Add New Sample** to create a unique sample name.

7. In the **Plate Setup** window, in the **Assign Targets and Samples** tab, confirm the following:
   - Wells containing the RNase P assay have only the RNaseP target assigned.
   - Wells containing the COVID-19 assay have all other targets assigned.

8. To assign a target or sample to a well, select the well, then check the **Assign** checkbox.

   IMPORTANT! Paired sample assay wells that do not have the same sample name will not be analyzed.

9. For wells with a positive control, confirm that **Task** is set to **S** for Standard.

10. For wells with a negative control, confirm that **Task** is set to **N** for Negative.

11. For wells with a patient sample, confirm that **Task** is set to **U** for Unknown.

12. Confirm that the **Passive Reference** is set to **None**.

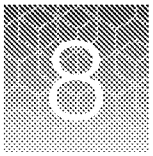


13. In the **Run Method** window, confirm that the **Reaction Volume Per Well** is 25 µL, then confirm the thermal protocol.

Step	Temperature	Time	Number of cycles
UNG incubation	25°C	2 minutes	1
Reverse transcription	53°C	10 minutes	1
Activation	95°C	2 minutes	1
Denaturation	95°C	3 seconds	40
Anneal / extension	60°C	30 seconds	

14. Select **Start Run**, enter a file name, then click **Save**.

15. After the instrument run is complete, click **Analyze**, then save the file.



Perform RT-PCR using the Applied Biosystems™ 7500 Real-Time PCR Instrument

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- Set up and run the 7500 Real-Time PCR Instrument—COVID-19 and RNase P assay 83

Dye calibration for the 7500 Real-Time PCR Instrument series

A maintained instrument will be calibrated for many dyes. In addition to those dyes, the instrument operator must calibrate the instrument for ABY™ dye and JUN™ dye that are used with this kit. For all other assays, refer to the standard calibration process.

IMPORTANT! Use only the calibration plates listed in "Required materials not supplied" on page 11.

Transfer the template (EDT) file for the 7500 Real-Time PCR Instrument

The template (EDT) file contains the settings for the instrument run. It is installed on the computer with Applied Biosystems™ COVID-19 Interpretive Software, and must be transferred via a USB drive or other method to the computer on which 7500 Software v2.3 is installed.

IMPORTANT! Be careful to select the appropriate template file for your instrument, software version, and assay type. Failure to do so can cause errors in the analysis.

1. On the computer with Applied Biosystems™ COVID-19 Interpretive Software, navigate to one of the following directories, based on whether the RT-PCR reaction contains only the COVID-19 assay, or both the COVID-19 assay and the RNase P assay (for Everlywell™ COVID-19 Test Home Collection Kit samples):

   - `<...>\Applied Biosystems\COVID-19 Interpretive Software\Client\docs\User Documents\COVID-19`
   - `<...>\Applied Biosystems\COVID-19 Interpretive Software\Client\docs\User Documents\COVID-19 + RNase P`

   <...> is the installation directory

2. Select the correct EDT file, based on whether the RT-PCR reaction contains only the COVID-19 assay, or both the COVID-19 assay and the RNase P assay (for Everlywell™ COVID-19 Test Home Collection Kit samples):

Assay type	Template name
COVID-19 only	`TaqPath COVID-19 Kit Template 7500std sds2_3 v1-2.edt`
COVID-19 and RNase P	`TaqPath COVID-19 and RNaseP Template 7500std sds2_3 v1-0.edt`

3. Transfer the EDT file to the computer with your data collection software, using a USB drive or other method.

Set up and run the 7500 Real-Time PCR Instrument—COVID-19 assay only

This section covers instrument set-up and operation for reaction plates that contain only the COVID-19 assay.

For plates that contain both the COVID-19 assay and RNase P assay, see "Set up and run the 7500 Fast Real-Time PCR Instrument (7500 Software v2.3)—COVID-19 plus RNase P assay" on page 77.

For more information about the instrument, see the documents listed in "Related documentation" on page 122.

1. In the 7500 Software v2.3 home page, click **Template**.
2. Browse to, then open the template file that you transferred in "Transfer the template (EDT) file for the 7500 Real-Time PCR Instrument" on page 81.

   IMPORTANT! Be careful to select the appropriate template file for your instrument, software version, and assay type. Failure to do so can cause errors in the analysis.
3. In the **Experiment Properties** window, enter or confirm the following information:
   - **Experiment name**: Enter a unique name
   - **Instrument type**: **7500 (96 wells)**
   - **Type of experiment**: **Quantitation - Standard Curve**
   - **Reagents**: **TaqMan™**
   - **Ramp Speed**: **Standard**
4. In the **Plate Setup** window, in the **Define Targets and Samples** tab and the **Define Targets** pane, confirm that the targets, reporter dyes, and quenchers are listed correctly.

Target	Reporter dye	Quencher
ORF1ab	FAM	None
N gene	VIC	None
S gene	ABY	None
MS2	JUN	None

5. In the **Plate Setup** window, in the **Define Targets and Samples** tab and the **Define Samples** pane, confirm the labeling of the control wells.
   - The template has one positive control (PC) and one negative control (NC) assigned to wells for reference.
   - Move the control well assignments by copying the existing control wells and pasting them according to their location on the physical plate.

   To include additional controls, select **Add New Sample**.
6. Select **Add New Sample** to assign a unique sample name to each well in the physical plate.

7. In the **Plate Setup** window, in the **Assign Targets and Samples** tab, confirm that four targets are assigned to each well in the plate layout. To designate a target or sample to a well, select the well, then check the **Assign** checkbox.

   Note: Wells that do not have a sample name will not be analyzed by the software.

8. For wells with a positive control, confirm that **Task** is set to **S** for Standard.

9. For wells with a negative control, confirm that **Task** is set to **N** for Negative.

10. For wells with a patient sample, confirm that **Task** is set to **U** for Unknown.

11. Confirm that the **Passive Reference** is set to **None**.

12. In the **Run Method** window, confirm that the **Reaction Volume Per Well** is 25 µL, then confirm the thermal protocol.

Step	Temperature	Time	Number of cycles
UNG incubation	25°C	2 minutes	1
Reverse transcription	53°C	10 minutes	1
Activation	95°C	2 minutes	1
Denaturation	95°C	3 seconds	40
Anneal / extension	60°C	30 seconds	

13. Select **Start Run**, enter a file name, then click **Save**.

14. After the instrument run is complete, click **Analyze**, then save the file.

Set up and run the 7500 Real-Time PCR Instrument—COVID-19 and RNase P assay

This section covers instrument set-up and operation for reaction plates that contain both the COVID-19 assay and the RNase P assay. Both assays are required for samples collected using the Everlywell™ COVID-19 Test Home Collection Kit.

For plates that contain only the COVID-19 assay, see "Set up and run the 7500 Real-Time PCR Instrument—COVID-19 assay only" on page 82.

For more information about the instrument, see the documents listed in "Related documentation" on page 122.

1. In the 7500 Software v2.3 home page, click **Template**.

2. Browse to, then open the template file that you transferred in "Transfer the template (EDT) file for the 7500 Real-Time PCR Instrument" on page 81.

   IMPORTANT! Be careful to select the appropriate template file for your instrument, software version, and assay type. Failure to do so can cause errors in the analysis.

3. In the **Experiment Properties** window, enter or confirm the following information:
   - **Experiment name**: Enter a unique name
   - **Instrument type**: **7500 (96 wells)**
   - **Type of experiment**: **Quantitation - Standard Curve**
   - **Reagents**: **TaqMan™**
   - **Ramp Speed**: **Standard**

4. In the **Plate Setup** window, in the **Define Targets and Samples** tab and the **Define Targets** pane, confirm that the targets, reporter dyes, and quenchers are listed correctly.

Target	Reporter dye	Quencher
COVID-19 assay targets		
ORF1ab	FAM	None
N gene	VIC	None
S gene	ABY	None
MS2	JUN	None
RNase P assay target		
RNaseP	VIC	None

5. In the **Plate Setup** window, in the **Define Targets and Samples** tab and the **Define Samples** pane, confirm the labeling of the control wells.
   - The template has one positive control and 2 negative controls (one each for the COVID-19 assay and RNase P assay) assigned to wells for reference.
   - Move the control well assignments by copying the existing control wells and pasting them according to their location on the physical plate.
   - To include additional controls, select **Add New Sample**.

6. Select **Add New Sample** to create a unique sample name.

7. In the **Plate Setup** window, in the **Assign Targets and Samples** tab, confirm the following:
   - Wells containing the RNase P assay have only the RNaseP target assigned.
   - Wells containing the COVID-19 assay have all other targets assigned.

8. To assign a target or sample to a well, select the well, then check the **Assign** checkbox.

   IMPORTANT! Each paired COVID-19 assay well and RNase P assay well must have the same sample name, or they will not be correctly analyzed by the software.

9. For wells with a positive control, confirm that **Task** is set to **S** for Standard.

10. For wells with a negative control, confirm that **Task** is set to **N** for Negative.

11. For wells with a patient sample, confirm that **Task** is set to **U** for Unknown.

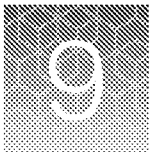
12. Confirm that the **Passive Reference** is set to **None**.

13. In the **Run Method** window, confirm that the **Reaction Volume Per Well** is 25 µL, then confirm the thermal protocol.

Step	Temperature	Time	Number of cycles
UNG incubation	25°C	2 minutes	1
Reverse transcription	53°C	10 minutes	1
Activation	95°C	2 minutes	1
Denaturation	95°C	3 seconds	40
Anneal / extension	60°C	30 seconds	

14. Select **Start Run**, enter a file name, then click **Save**.

15. After the instrument run is complete, click **Analyze**, then save the file.



Perform RT-PCR using the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR Instrument

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- Transfer the template (EDT) file for the QuantStudio™ 5 Real-Time PCR Instrument 87
- Set up and run the QuantStudio™ 5 Real-Time PCR Instrument (96-well plates)—COVID-19 assay only 88
- Set up and run the QuantStudio™ 5 Real-Time PCR Instrument (384-well plates)—COVID-19 assay only 90
- Set up and run the QuantStudio™ 5 Real-Time PCR Instrument (96-well plates)—COVID-19 and RNase P assay 92
- Set up and run the QuantStudio™ 5 Real-Time PCR Instrument (384-well plates)—COVID-19 and RNase P assay 94

Dye calibration for the QuantStudio™ 5 Real-Time PCR Instrument

A maintained instrument will be calibrated for all dyes that are used with this kit. Ensure that the calibrations for FAM™ dye, VIC™ dye, ABY™ dye, and JUN™ dye are current. If calibration is required, refer to the standard calibration process in the instrument user guide.



Transfer the template (EDT) file for the QuantStudio™ 5 Real-Time PCR Instrument

The template (EDT) file contains the settings for the instrument run. It is installed on the computer with Applied Biosystems™ COVID-19 Interpretive Software, and must be transferred via a USB drive or other method to the computer on which QuantStudio™ Design and Analysis Desktop Software v1.5.1 is installed.

IMPORTANT! Be careful to select the appropriate template file for your instrument, block type, and assay type. Failure to do so can cause errors in the analysis.

---

1. On the computer with Applied Biosystems™ COVID-19 Interpretive Software, navigate to one of the following directories, based on whether the RT-PCR reaction contains only the COVID-19 assay, or both the COVID-19 assay and the RNase P assay (for Everlywell™ COVID-19 Test Home Collection Kit samples):

   - `<...>\Applied Biosystems\COVID-19 Interpretive Software\Client\docs\User Documents\COVID-19`
   - `<...>\Applied Biosystems\COVID-19 Interpretive Software\Client\docs\User Documents\COVID-19 + RNase P`

   <...> is the installation directory

2. Select the correct template file, based on whether the RT-PCR reaction contains only the COVID-19 assay, or both the COVID-19 assay and RNase P assay (for Everlywell™ COVID-19 Test Home Collection Kit samples):

Table 23 COVID-19 assay only

Instrument	Template file
QuantStudio™ 5 Real-Time PCR Instrument (96-well, 0.1-mL block)	`TaqPath COVID-19 Kit Template QS5 0_1ml_da1_5_1 v2-2.edt`
QuantStudio™ 5 Real-Time PCR Instrument (96-well, 0.2-mL block)	`TaqPath COVID-19 Kit Template QS5 0_2ml_da1_5_1 v2-2.edt`
QuantStudio™ 5 Real-Time PCR Instrument (384-well block)	`TaqPath COVID-19 Kit Template QS5 384_da1_5_1 v2-2.edt`

Table 24 COVID-19 assay and RNase P assay

Instrument	Template file
QuantStudio™ 5 Real-Time PCR Instrument (96-well, 0.1-mL block)	`TaqPath COVID-19 and RNaseP Template QS5 0_1ml_da1_5_1 v1-0.edt`
QuantStudio™ 5 Real-Time PCR Instrument (96-well, 0.2-mL block)	`TaqPath COVID-19 and RNaseP Template QS5 0_2ml_da1_5_1 v1-0.edt`
QuantStudio™ 5 Real-Time PCR Instrument (384-well block)	`TaqPath COVID-19 and RNaseP Template QS5 384_da1_5_1 v1-0.edt`

3. Transfer the EDT file to the computer with QuantStudio™ Design and Analysis Desktop Software v1.5.1, using a USB drive or other method.

Set up and run the QuantStudio™ 5 Real-Time PCR Instrument (96-well plates)—COVID-19 assay only

This section covers instrument set-up and operation for reaction plates that contain only the COVID-19 assay.

For 96-well plates that contain both the COVID-19 assay and RNase P assay, see "Set up and run the QuantStudio™ 5 Real-Time PCR Instrument (96-well plates)—COVID-19 and RNase P assay" on page 92.

For more information about the instrument, see the documents listed in "Related documentation" on page 122.

1. In the QuantStudio™ Design and Analysis Desktop Software v1.5.1, in the **New Experiment** box, select **Create New Experiment** ▸ **Template**.

2. Browse to, then open the EDT file that you transferred in "Transfer the template (EDT) file for the QuantStudio™ 5 Real-Time PCR Instrument" on page 87.

   IMPORTANT! Be careful to select the appropriate template file for your instrument and block type. Failure to do so can cause errors in the analysis.

   Be careful to select the appropriate template file for your instrument, block type, and assay type. Failure to do so can cause errors in the analysis.

3. In the **Properties** tab, enter or confirm the following.

   - **Name**: Enter a unique name
   - **Instrument type**: **QuantStudio™ 5 System**
   - **Block type**: **96-Well 0.2-mL Block** *or* **96-Well 0.1-mL Block**
   - **Experiment type**: **Standard Curve**
   - **Chemistry**: **TaqMan™ Reagents**
   - **Run Mode**: **Standard**

4. In the **Method** tab, confirm that the **Volume** is 25 µL, then confirm the thermal protocol.

Step	Temperature	Time	Number of cycles
UNG incubation	25°C	2 minutes	1
Reverse transcription	53°C	10 minutes	1
Activation	95°C	2 minutes	1
Denaturation	95°C	3 seconds	40
Anneal / extension	60°C	30 seconds	

5. In the **Plate** tab, click **Quick Setup**.

6. In the **Plate Attributes** pane, confirm that the **Passive Reference** is set to **None**.

7. In the **Plate** tab, click **Advanced Setup**.

8. In the **Targets** table, confirm that the reporter dye and the target pairs are correct.

Reporter dye	Detector	Quencher
FAM	ORF1ab	None
VIC	N gene	None
ABY	S gene	None
JUN	MS2	None

9. Confirm that the targets above are assigned to each well in the plate layout.

10. In the plate layout pane, confirm the labeling of the control wells.
    - The template has one positive control (PC) and one negative control (NC) assigned to wells for reference.
    - Move the control well assignments by copying the existing control wells and pasting them according to their location on the physical plate.

11. For all targets in the positive control well, confirm that **Task** is set to **S (Standard)**.

12. For all targets in the negative control well, confirm that **Task** is set to **N (Negative Control)**.

13. In the **Samples** table, click **Add** to define the sample names. Create a unique sample name for each well in the physical plate.

14. To assign a sample to a well, select the well in the plate layout, then select the sample from the **Samples** table.

    For all targets in the patient sample wells, confirm that **Task** is set to **U (Unknown)**.

Note: Wells that do not have a sample name will not be analyzed by the software.

15. In the **Run** tab, click **Start Run**, then select your instrument from the drop-down list.

16. Enter a file name in the dialog box that prompts you to save the run file, then save the file.

Set up and run the QuantStudio™ 5 Real-Time PCR Instrument (384-well plates)—COVID-19 assay only

This section covers instrument set-up and operation for reaction plates that contain only the COVID-19 assay.

For plates that contain both the COVID-19 assay and RNase P assay, see "Set up and run the QuantStudio™ 5 Real-Time PCR Instrument (384-well plates)—COVID-19 and RNase P assay" on page 94.

For more information about the instrument, see the documents listed in "Related documentation" on page 122.

1. In the QuantStudio™ Design and Analysis Desktop Software v1.5.1, in the **New Experiment** box, select **Create New Experiment ▸ Template**.

2. Browse to, then open the EDT file that you transferred in "Transfer the template (EDT) file for the QuantStudio™ 5 Real-Time PCR Instrument" on page 87.

   IMPORTANT! Be careful to select the appropriate template file for your instrument, block type, and assay type. Failure to do so can cause errors in the analysis.

3. In the **Properties** tab, enter or confirm the following.
   - **Name**: Enter a name
   - **Instrument type**: **QuantStudio™ 5 System**
   - **Block type**: **384-well Block**
   - **Experiment type**: **Standard Curve**
   - **Chemistry**: **TaqMan™ Reagents**
   - **Run Mode**: **Standard**

4. In the **Method** tab, confirm that the **Volume** is 20 µL, then confirm the thermal protocol.

Step	Temperature	Time	Number of cycles
UNG incubation	25°C	2 minutes	1
Reverse transcription	53°C	10 minutes	1
Activation	95°C	2 minutes	1
Denaturation	95°C	3 seconds	40
Anneal / extension	60°C	30 seconds	

5. In the **Plate** tab, click **Quick Setup**.

6. In the **Plate Attributes** pane, confirm that the **Passive Reference** is set to **None**.

7. In the **Plate** tab, click **Advanced Setup**.

8. In the **Targets** table, confirm that the reporter dye and the target pairs are correct.

Reporter dye	Detector	Quencher
FAM	ORF1ab	None
VIC	N gene	None
ABY	S gene	None
JUN	MS2	None

9. Confirm that the targets above are assigned to each well in the plate layout.

10. In the plate layout pane, confirm the labeling of the control wells.
    - The template has one positive control (PC) and one negative control (NC) assigned to wells for reference.
    - Move the control well assignments by copying the existing control wells and pasting them according to their location on the physical plate.

Note: If samples from more than one RNA extraction run are being included on the same RT-PCR 384-well plate, then there must be one negative control for each RNA extraction run included on the RT-PCR 384-well plate (see "Guidelines for RT-PCR" on page 35). Label each negative control with a unique name, for example, ***NC1***, ***NC2***, ***NC3***, and ***NC4***.

---

11. For all targets in the positive control well, confirm that **Task** is set to **S (Standard)**.

12. For all targets in the negative control well, confirm that **Task** is set to **N (Negative Control)**.

13. In the **Samples** table, click **Add** to define the names. Create a unique sample name for each well in the physical plate.

    The template has one positive control and one negative control assigned to wells for reference. If additional control wells are required, each control well must have a unique name.

14. To assign a sample to a well, select the well in the plate layout, then select the sample from the **Samples** table.

    For all targets in the patient sample wells, confirm that **Task** is set to **U (Unknown)**.

Note: Wells that do not have a sample name will not be analyzed by the software.

---

15. In the **Run** tab, click **Start Run**, then select your instrument from the drop-down list.

16. Enter a file name in the dialog box that prompts you to save the run file, then save the file.

Set up and run the QuantStudio™ 5 Real-Time PCR Instrument (96-well plates)—COVID-19 and RNase P assay

This section covers instrument set-up and operation for 96-well reaction plates that contain both the COVID-19 assay and the RNase P assay. Both assays are required for samples collected using the Everlywell™ COVID-19 Test Home Collection Kit.

For 96-well plates that contain only the COVID-19 assay, see "Set up and run the QuantStudio™ 5 Real-Time PCR Instrument (96-well plates)—COVID-19 assay only" on page 88.

For more information about the instrument, see the documents listed in "Related documentation" on page 122.

1. In the QuantStudio™ Design and Analysis Desktop Software v1.5.1, in the **New Experiment** box, select **Create New Experiment ▸ Template**.

2. Browse to, then open the EDT file that you transferred in "Transfer the template (EDT) file for the QuantStudio™ 5 Real-Time PCR Instrument" on page 87.

   IMPORTANT! Be careful to select the appropriate template file for your instrument, software version, and assay type. Failure to do so can cause errors in the analysis.

3. In the **Properties** tab, enter or confirm the following.
   - **Name**: Enter a unique name
   - **Instrument type**: **QuantStudio™ 5 System**
   - **Block type**: **96-Well 0.2-mL Block** *or* **96-Well 0.1-mL Block**
   - **Experiment type**: **Standard Curve**
   - **Chemistry**: **TaqMan™ Reagents**
   - **Run Mode**: **Standard**

4. In the **Method** tab, confirm that the **Volume** is 25 µL, then confirm the thermal protocol.

Step	Temperature	Time	Number of cycles
UNG incubation	25°C	2 minutes	1
Reverse transcription	53°C	10 minutes	1
Activation	95°C	2 minutes	1
Denaturation	95°C	3 seconds	40
Anneal / extension	60°C	30 seconds	

5. In the **Plate** tab, click **Quick Setup**.

6. In the **Plate Attributes** pane, confirm that the **Passive Reference** is set to **None**.

7. In the **Plate** tab, click **Advanced Setup**.

8. In the **Targets** table, confirm that the reporter dye and the target pairs are correct.

Reporter dye	Detector	Quencher
COVID-19 assay targets		
FAM	ORF1ab	None
VIC	N gene	None
ABY	S gene	None
JUN	MS2	None
RNase P assay target		
VIC	RNaseP	None

9. Confirm that the targets above are assigned to each well in the plate layout.

Note:
- Wells containing the RNase P assay have only the RNaseP detector assigned.
- Wells containing the COVID-19 assay have all other detectors assigned.

10. In the plate layout pane, confirm the labeling of the control wells.
    - The template has one positive control and 2 negative controls (one each for the COVID-19 assay and RNase P assay) assigned to wells for reference.
    - Move the control well assignments by copying the existing control wells and pasting them according to their location on the physical plate.

11. For all targets in the positive control well, confirm that **Task** is set to **S (Standard)**.

12. For all targets in the negative control well, confirm that **Task** is set to **N (Negative Control)**.

13. In the **Samples** table, click **Add** to define the sample names. The same sample name is used for each paired COVID-19 assay well and RNase P assay well in the physical plate.

14. To assign a sample to a well, select the well in the plate layout, then select the sample from the **Samples** table.

IMPORTANT! Paired sample assay wells that do not have the same sample name will not be analyzed by the software.

For all targets in the patient sample wells, confirm that **Task** is set to **U (Unknown)**.

15. In the **Run** tab, click **Start Run**, then select your instrument from the drop-down list.

16. Enter a file name in the dialog box that prompts you to save the run file, then save the file.

Set up and run the QuantStudio™ 5 Real-Time PCR Instrument (384-well plates)—COVID-19 and RNase P assay

This section covers instrument set-up and operation for 384-well reaction plates that contain both the COVID-19 assay and the RNase P assay. Both assays are required for samples collected using the Everlywell™ COVID-19 Test Home Collection Kit.

For 384-well plates that contain only the COVID-19 assay, see "Set up and run the QuantStudio™ 5 Real-Time PCR Instrument (384-well plates)—COVID-19 assay only" on page 90.

For more information about the instrument, see the documents listed in "Related documentation" on page 122.

1. In the QuantStudio™ Design and Analysis Desktop Software v1.5.1, in the **New Experiment** box, select **Create New Experiment ▸ Template**.

2. Browse to, then open the EDT file that you transferred in "Transfer the template (EDT) file for the QuantStudio™ 5 Real-Time PCR Instrument" on page 87.

   IMPORTANT! Be careful to select the appropriate template file for your instrument, block type, and assay type. Failure to do so can cause errors in the analysis.

3. In the **Properties** tab, enter or confirm the following.
   - **Name**: Enter a name
   - **Instrument type**: **QuantStudio™ 5 System**
   - **Block type**: **384-well Block**
   - **Experiment type**: **Standard Curve**
   - **Chemistry**: **TaqMan™ Reagents**
   - **Run Mode**: **Standard**

4. In the **Method** tab, confirm that the **Volume** is 20 µL, then confirm the thermal protocol.

Step	Temperature	Time	Number of cycles
UNG incubation	25°C	2 minutes	1
Reverse transcription	53°C	10 minutes	1
Activation	95°C	2 minutes	1
Denaturation	95°C	3 seconds	40
Anneal / extension	60°C	30 seconds	

5. In the **Plate** tab, click **Quick Setup**.

6. In the **Plate Attributes** pane, confirm that the **Passive Reference** is set to **None**.

7. In the **Plate** tab, click **Advanced Setup**.

8. In the **Targets** table, confirm that the reporter dye and the target pairs are correct.

Reporter dye	Detector	Quencher
COVID-19 assay targets		
FAM	ORF1ab	None
VIC	N gene	None
ABY	S gene	None
JUN	MS2	None
RNase P assay target		
VIC	RNaseP	None

9. Confirm that the targets above are assigned to each well in the plate layout.

Note:
- Wells containing the RNase P assay have only the RNaseP detector assigned.
- Wells containing the COVID-19 assay have all other detectors assigned.

10. In the plate layout pane, confirm the labeling of the control wells.
    - The template has one positive control and 2 negative controls (one each for the COVID-19 assay and RNase P assay) assigned. To assign additional negative control wells, see step 15.
    - Move the control well assignments by copying the existing control wells and pasting them according to their location on the physical plate.

11. For all targets in the positive control well, confirm that **Task** is set to **S (Standard)**.

12. For all targets in each negative control well, confirm that **Task** is set to **N (Negative Control)**.

13. In the **Samples** table, click **Add** to define the sample names.

    The same sample name is used for each paired COVID-19 assay well and RNase P assay well in the physical plate.

14. To assign a sample to paired COVID-19 and RNase P assay wells, select each well in the plate layout, then select the sample from the **Samples** table.

IMPORTANT! Paired sample assay wells that do not have the same name will not be analyzed by the software.

    For all targets in the patient sample wells, confirm that **Task** is set to **U (Unknown)**.

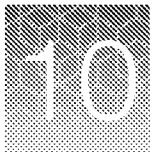
15. If the 384-well plate includes additional negative controls (from combined extraction runs), define each additional negative control and assign it to paired control wells (COVID-19 and RNase P) to match the physical plate.

    Give each negative control a unique name (for example, ***NC1***, ***NC2***, and so forth).



16. In the **Run** tab, click **Start Run**, then select your instrument from the drop-down list.

17. Enter a file name in the dialog box that prompts you to save the run file, then save the file.



Perform RT-PCR using the Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR Instrument (384-well block)

Dye calibration for the QuantStudio™ 7 Flex Real-Time PCR Instrument

A maintained instrument will be calibrated for many dyes. In addition to those dyes, the instrument operator must calibrate the instrument for ABY™ dye and JUN™ dye that are used with this kit. For all other assays, refer to the standard calibration process.

Transfer the template (EDT) file for the QuantStudio™ 7 Flex Real-Time PCR Instrument (384–well block)

The template (EDT) file contains the settings for the instrument run. It is installed on the computer with Applied Biosystems™ COVID-19 Interpretive Software, and must be transferred via a USB drive or other method to the computer on which the QuantStudio™ Real-Time PCR Software v1.3 is installed.

IMPORTANT! Be careful to select the appropriate template file for your instrument, block type, and assay type. Failure to do so can cause errors in the analysis.

---

1. On the computer with Applied Biosystems™ COVID-19 Interpretive Software, navigate to one of the following directories, based on whether the RT-PCR reaction contains only the COVID-19 assay, or both the COVID-19 assay and the RNase P assay (for Everlywell™ COVID-19 Test Home Collection Kit samples):

   - `<...>\Applied Biosystems\COVID-19 Interpretive Software\Client\docs\User Documents\COVID-19`
   - `<...>\Applied Biosystems\COVID-19 Interpretive Software\Client\docs\User Documents\COVID-19 + RNase P`

   <...> is the installation directory



2. Select the correct EDT file, based on whether the RT-PCR reaction contains only the COVID-19 assay, or both the COVID-19 assay and the RNase P assay (for Everlywell™ COVID-19 Test Home Collection Kit samples):

Assay type	Template name
COVID-19 only	`TaqPath COVID-19 Kit Template QS7 384 1_3 v2-2.edt`
COVID-19 and RNase P	`TaqPath COVID-19 and RNaseP Template QS7 384 1_3 v1-0.edt`

3. Transfer the EDT file to the computer with QuantStudio™ Real-Time PCR Software v1.3, using a USB drive or other method.

Set up and run the QuantStudio™ 7 Flex Real-Time PCR Instrument (384–well block)—COVID-19 assay only

This section covers instrument set-up and operation for reaction plates that contain only the COVID-19 assay.

For plates that contain both the COVID-19 assay and RNase P assay, see "Set up and run the QuantStudio™ 7 Flex Real-Time PCR Instrument (384–well block)—COVID-19 and RNase P assay" on page 100.

For more information about the instrument, see the documents listed in "Related documentation" on page 122.

1. In the QuantStudio™ Real-Time PCR Software v1.3 home screen, click **Template**.

2. Browse to, then open the EDT file that you transferred in "Transfer the template (EDT) file for the QuantStudio™ 7 Flex Real-Time PCR Instrument (384–well block)" on page 97.

   IMPORTANT! Be careful to select the appropriate template file for your instrument, block type, and assay type. Failure to do so can cause errors in the analysis.

3. In the **Experiment Properties** tab, enter or confirm the following.
   - **Experiment Name**: Enter a unique name
   - **Instrument type**: **QuantStudio™ 7 Flex System**
   - **Block**: **384-well**
   - **Type of Experiment**: **Standard Curve**
   - **Reagents**: **TaqMan™**
   - **Properties**: **Standard**

4. In the **Define** tab, in the **Targets** pane, confirm that the targets, reporter dyes, and quenchers are listed correctly.

Target	Reporter dye	Quencher
ORF1ab	FAM	None
N gene	VIC	None
S gene	ABY	None
MS2	JUN	None

5. In the **Define** tab, in the **Samples** pane, define a unique sample name for each well in the physical plate.

Note: Wells that do not have a sample name will not be analyzed by the software.

6. In the **Define** tab, confirm that the **Passive Reference** is set to **None**.

7. In the **Assign** tab, confirm that four targets are assigned to each well in the plate layout.
   To assign a target to a well, select the well, then check the **Assign** checkbox.

8. In the **Assign** tab, in the **Samples** pane, confirm the labeling of the control wells.
   - The template has one positive control (PC) and one negative control (NC) assigned to wells for reference.
   - Move the control well assignments by copying the existing control wells and pasting them according to their location on the physical plate.

Note: If the 384-well plate includes additional negative controls (from combined extraction runs), define each additional negative control and assign it to a well to match the physical plate. Give each negative control a unique name (for example, ***NC1***, ***NC2***, ***NC3***, and ***NC4***).

9. In the **Assign** tab, confirm the **Task** assignments.
   - For wells with a Positive Control (PC), confirm that the **Task** is set to **S** for Standard for all of the targets.
   - For wells with a Negative Control (NC), confirm that the **Task** is set to **N** for Negative for all of the targets.
   - For the wells with a patient sample, confirm that the **Task** is set to **U** for Unknown for all of the targets.

10. In the **Assign** tab, assign a sample name to each well to match the physical plate.
    To assign a sample to a well, select the well, then check the **Assign** checkbox.



11. In the **Run Method** tab, confirm that the **Reaction Volume Per Well** is 20 µL, then confirm the thermal protocol.

Step	Temperature	Time	Number of cycles
UNG incubation	25°C	2 minutes	1
Reverse transcription	53°C	10 minutes	1
Activation	95°C	2 minutes	1
Denaturation	95°C	3 seconds	40
Anneal / extension	60°C	30 seconds	

12. In the **Run** tab, click **Start Run**, then select your instrument from the drop-down list.

13. Enter a file name in the dialog box that prompts you to save the run file, then save the file.

Set up and run the QuantStudio™ 7 Flex Real-Time PCR Instrument (384–well block)—COVID-19 and RNase P assay

This section covers instrument set-up and operation for 384-well reaction plates that contain both the COVID-19 assay and the RNase P assay. Both assays are required for samples collected using the Everlywell™ COVID-19 Test Home Collection Kit.

For 384-well plates that contain only the COVID-19 assay, see "Set up and run the QuantStudio™ 7 Flex Real-Time PCR Instrument (384–well block)—COVID-19 assay only" on page 98.

For more information about the instrument, see the documents listed in "Related documentation" on page 122.

1. In the QuantStudio™ Real-Time PCR Software v1.3 home screen, click **Template**.

2. Browse to, then open the EDT file that you transferred in "Transfer the template (EDT) file for the QuantStudio™ 7 Flex Real-Time PCR Instrument (384–well block)" on page 97.

   IMPORTANT! Be careful to select the appropriate template file for your instrument, block type, and assay type. Failure to do so can cause errors in the analysis.

3. In the **Experiment Properties** tab, enter or confirm the following.

   - **Experiment Name**: Enter a unique name
   - **Instrument type**: **QuantStudio™ 7 Flex System**
   - **Block**: **384-well**
   - **Type of Experiment**: **Standard Curve**
   - **Reagents**: **TaqMan™**
   - **Properties**: **Standard**

4. In the **Define** tab, in the **Targets** pane, confirm that the targets, reporter dyes, and quenchers are listed correctly.

Target	Reporter dye	Quencher
COVID-19 assay targets		
ORF1ab	FAM	None
N gene	VIC	None
S gene	ABY	None
MS2	JUN	None
RNase P assay target		
RNaseP	VIC	None

5. In the **Define** tab, in the **Samples** pane, define a sample name for each sample in the physical plate.

   The same sample name is used for each paired COVID-19 assay well and RNase P assay well in the physical plate.

   IMPORTANT! Paired sample assay wells that do not have the same name will not be analyzed by the software.

6. In the **Define** tab, confirm that the **Passive Reference** is set to **None**.

7. In the **Assign** tab, confirm that targets are assigned to each well in the plate layout.

   Note:
   - Wells containing the RNase P assay have only the RNaseP target assigned.
   - Wells containing the COVID-19 assay have all other targets assigned.

   To assign a target to a well, select the well, then check the **Assign** checkbox.

8. In the **Assign** tab, in the **Samples** pane, confirm the labeling of the control wells.
   - The template has one positive control and 2 negative controls (one each for the COVID-19 assay and RNase P assay) assigned to wells for reference.
   - Move the control well assignments by copying the existing control wells and pasting them according to their location on the physical plate.

   Note: If the 384-well plate includes additional negative controls (from combined extraction runs), define each additional negative control and assign it to paired control wells (COVID-19 and RNase P) to match the physical plate.

   Give each negative control a unique name (for example, ***NC1***, ***NC2***, and so forth).



9. In the **Assign** tab, confirm the **Task** assignments.
   - For wells with a Positive Control (PC), confirm that the **Task** is set to **S** for Standard for all of the targets.
   - For wells with a Negative Control (NC), confirm that the **Task** is set to **N** for Negative for all of the targets.
   - For the wells with a patient sample, confirm that the **Task** is set to **U** for Unknown for all of the targets.

10. In the **Assign** tab, assign a sample name to paired wells (COVID-19 and RNase P) to match the physical plate.

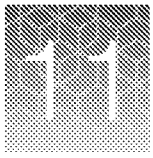
    To assign a sample to a well, select the well, then check the **Assign** checkbox.

11. In the **Run Method** tab, confirm that the **Reaction Volume Per Well** is 20 µL, then confirm the thermal protocol.

Step	Temperature	Time	Number of cycles
UNG incubation	25°C	2 minutes	1
Reverse transcription	53°C	10 minutes	1
Activation	95°C	2 minutes	1
Denaturation	95°C	3 seconds	40
Anneal / extension	60°C	30 seconds	

12. In the **Run** tab, click **Start Run**, then select your instrument from the drop-down list.

13. Enter a file name in the dialog box that prompts you to save the run file, then save the file.



Analysis and results

Obtain the Applied Biosystems™ COVID-19 Interpretive Software

To perform data analysis and results interpretation, you must use the Applied Biosystems™ COVID-19 Interpretive Software.

To obtain the software, contact your local instrument service team. Go to https://www.thermofisher.com/contactus.

Analyze the data

For detailed instructions about using the software, click the **Help** menu in the COVID-19 Interpretive Software.

1. Using a USB drive or other method, transfer the SDS or EDS files from the computer with the data collection software to the computer with the COVID-19 Interpretive Software.
2. Sign in to the COVID-19 Interpretive Software, then click the button that is appropriate for your test:
   - **COVID-19 Test**
   - **COVID-19 + RNase P Test**
3. In the **Home** screen, click the **Import Samples** button.
4. Select the SDS files or the EDS files to import, then click **Open**.
   After import, the software analyzes the run data, performs Quality Check (QC) analysis, and calculates the interpretive results for each sample and control.
5. In the **Home** screen, under **Batches**, click the `<Batch ID>` link for the batch of interest to display the **Batch Details** screen.
   - In the **Batch Details** screen, view the status and result for each sample in the **Sample Information** tab.
   - **Optional:** To identify signs of inadequate vortexing or centrifugation, select the **Amplification Plot** tab to view the amplification curve for each sample.
   - **Optional:** To enter a comment for the sample, select the **Amplification Plot** tab, select the **Comment** tab, enter a comment, then click **Add**.
     The comment will appear in all exports (CSV files) and reports (PDF files). It will include the user name and date.

6. To generate a Batch Export (CSV file), return to the **Home** screen, select the checkbox for the batch, then click **Export Batch** at the top of the **Home** screen. Click **Open folder location** in the dialog box to locate and open the CSV file.

7. To generate a Batch Report (PDF file), select the checkbox for the batch, then click **Report Batch** at the top of the **Home** screen. Click **Open folder location** in the dialog box to locate and open the PDF file.

Interpretation of the results

Interpretation of the results is performed by the Applied Biosystems™ COVID-19 Interpretive Software. For information about the C_t values that are used by the software to interpret results, see Appendix B, "Ct cutoff values for assay targets".

Quality control and validity of results

A minimum of one Negative Control and one Positive Control must be present for each run for the COVID-19 assay.

A minimum of two Negative Controls (one for the COVID-19 assay and one for the RNase P assay) and one Positive Control must be present for each run for the COVID-19 plus RNase P assay.

Additional Negative Control wells must be run for each extraction that is represented on a real-time RT-PCR plate. All control wells must pass for the real-time RT-PCR plate to be considered valid.

Validation of results is performed automatically by the Applied Biosystems™ COVID-19 Interpretive Software based on performance of the Positive and Negative Controls.

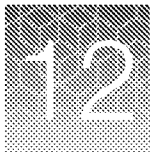
Table 25 COVID-19 Test—Patient samples

ORF1ab	N gene	S gene	MS2	Status	Result	Action
NEG	NEG	NEG	NEG	INVALID	NA	Repeat test by re-extracting the original sample and repeating the RT-PCR. If the repeat result remains invalid, consider collecting a new specimen.
NEG	NEG	NEG	POS	VALID	SARS-CoV-2 Not Detected	Report results to the healthcare provider and appropriate public health authorities. Consider testing for other viruses.
Only one SARS-CoV-2 target = POS			POS or NEG	VALID	SARS-CoV-2 Inconclusive	1. Repeat test by re-extracting the original sample and repeating the RT-PCR. 2. After retesting one time, report results to the healthcare provider and appropriate public health authorities. **IMPORTANT!** Samples with a result of SARS-CoV-2 Inconclusive shall be retested one time. If the repeat result remains inconclusive, the healthcare provider should conduct additional confirmation testing with a new specimen, if clinically indicated.
Two or more SARS-CoV-2 targets = POS			POS or NEG	VALID	Positive SARS-CoV-2	Report results to the healthcare provider and appropriate public health authorities.

Table 26 COVID-19 + RNase P Test—Patient samples

ORF1ab	N gene	S gene	MS2	RNase P	Status	Result	Action
NEG	NEG	NEG	NEG	NEG	INVALID	NA	Repeat test by re-extracting the original sample and repeating the RT-PCR. If the repeat result remains invalid, consider collecting a new specimen.
NEG	NEG	NEG	NEG	POS	INVALID	NA	Repeat test by re-extracting the original sample and repeating the RT-PCR. If the repeat result remains invalid, consider collecting a new specimen.
NEG	NEG	NEG	POS	POS	VALID	SARS-CoV-2 Not Detected	Report results to the healthcare provider and appropriate public health authorities. Consider testing for other viruses.
NEG	NEG	NEG	POS	NEG	INVALID	NA	Repeat test by re-extracting the original sample and repeating the RT-PCR. If the repeat result remains invalid, consider collecting a new specimen.
Only one SARS-CoV-2 target = POS			POS or NEG		VALID	SARS-CoV-2 Inconclusive[1]	1. Repeat test by re-extracting the original sample and repeating the RT-PCR. 2. After retesting one time, report results to the healthcare provider and appropriate public health authorities. **IMPORTANT!** Samples with a result of SARS-CoV-2 Inconclusive shall be retested one time. If the repeat result remains inconclusive, the healthcare provider should conduct additional confirmation testing with a new specimen, if clinically indicated.
Two or more SARS-CoV-2 targets = POS			POS or NEG		VALID	Positive SARS-CoV-2	Report results to the healthcare provider and appropriate public health authorities.

[1] Samples with a result of SARS-CoV-2 Inconclusive shall be retested one time.



Conditions of authorization for labs

The TaqPath™ COVID-19 RT-PCR Kit and TaqPath™ COVID-19 Combo Kit Advanced Letter of Authorization, along with the authorized Fact Sheet for Healthcare Providers, the authorized Fact Sheet for Patients, and authorized labeling are available on the FDA website: https://www.fda.gov/emergency-preparedness-and-response/mcm-legal-regulatory-and-policy-framework/emergency-use-authorization#2019-ncov.

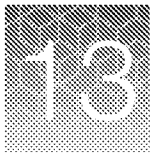
However, to assist clinical laboratories using the TaqPath™ COVID-19 RT-PCR Kit and the TaqPath™ COVID-19 RT-PCR Kit Advanced, the relevant Conditions of Authorization are listed below.

- Authorized laboratories[1] using the TaqPath™ COVID-19 Combo Kit and the TaqPath™ COVID-19 Combo Kit Advanced will include with result reports of the TaqPath™ COVID-19 Combo Kit and the TaqPath™ COVID-19 Combo Kit Advanced all authorized Fact Sheets. Under exigent circumstances, other appropriate methods for disseminating these Fact Sheets may be used, which may include mass media.
- Authorized laboratories using the TaqPath™ COVID-19 Combo Kit and the TaqPath™ COVID-19 Combo Kit Advanced will perform the TaqPath™ COVID-19 Combo Kit and the TaqPath™ COVID-19 Combo Kit Advanced as outlined in the *TaqPath™ COVID-19 Combo Kit and TaqPath™ COVID-19 Combo Kit Advanced Instructions for Use*. Deviations from the authorized procedures, including the authorized instruments, authorized extraction methods, authorized clinical specimen types, authorized control materials, authorized other ancillary reagents, and authorized materials required to perform the TaqPath™ COVID-19 Combo Kit and the TaqPath™ COVID-19 Combo Kit Advanced are not permitted.
- Authorized laboratories that receive the TaqPath™ COVID-19 Combo Kit and the TaqPath™ COVID-19 Combo Kit Advanced must notify the relevant public health authorities of their intent to run the test prior to initiating testing.
- Authorized laboratories using the TaqPath™ COVID-19 Combo Kit and the TaqPath™ COVID-19 Combo Kit Advanced will have a process in place for reporting test results to healthcare providers and relevant public health authorities, as appropriate.
- Authorized laboratories testing nasal swab specimens self-collected using using the Everlywell™ COVID-19 Test Home Collection Kit must be laboratories designated by Everlywell, Inc. and follow the *Receiving and Processing Everlywell Samples* standard operating procedure when accepting specimens for testing.
- Authorized laboratories will collect information on the performance of the test and report to DMD/OHT7-OIR/OPEQ/CDRH (via email: CDRH-EUA-Reporting@fda.hhs.gov) and Thermo Fisher Scientific (lechservices@thermofisher.com; 1 800 955 6288) any suspected occurrence of false positive or false negative results and significant deviations from the established performance characteristics of the test of which they become aware.

[1] For ease of reference, this letter will refer to, "Laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, that meet requirements to perform high complexity tests" as "authorized laboratories."



- All laboratory personnel using the test must be appropriately trained in RT-PCR techniques and use appropriate laboratory and personal protective equipment when handling this kit, and use the test in accordance with the authorized labeling.
- Thermo Fisher Scientific, its authorized distributor(s), and authorized laboratories using the TaqPath™ COVID-19 Combo Kit and the TaqPath™ COVID-19 Combo Kit Advanced will ensure that any records associated with this EUA are maintained until otherwise notified by FDA. Such records will be made available to FDA for inspection upon request.



Performance characteristics

Analytical performance of the TaqPath™ COVID-19 RT-PCR Kit was evaluated by determining limit of detection (LoD), characterizing the impact of interfering substances and cross-reactivity, as described in the following sections.

Limit of detection (LoD)

The LoD study established the lowest SARS-CoV-2 viral concentration (Genomic Copy Equivalents or GCE) that can be detected by the TaqPath™ COVID-19 Combo Kit in a particular specimen type at least 95% of the time. Banked Nasopharyngeal swab (NP) and Bronchoalveolar lavage (BAL) samples, obtained from U.S. patients in the years 2015-2019, were pooled, respectively, and spiked with purified SARS-CoV-2 viral genomic RNA at several concentrations and processed through the TaqPath™ COVID-19 Combo Kit workflow. A three-phase approach was used to determine the LoD for each specimen type. In phases I and II, the preliminary LoD was established and confirmed in phase III by testing 20 replicates.

Table 27 LoD determination in BAL

Effective Concentration	Replicate	Mean C_t				Interpretation	% Positive
		ORF1ab	N	S	MS2		
10 GCE/reaction	1	29.9	29.1	28.5	23.1	Positive	100%
	2	30.1	29.3	29.7	24.0	Positive	
	3	30.0	29.7	29.3	24.0	Positive	
	4	30.3	29.7	29.1	23.8	Positive	
	5	30.2	29.6	29.6	23.7	Positive	
	6	30.3	29.3	29.7	23.5	Positive	
	7	29.9	29.6	32.8	23.4	Positive	
	8	30.2	29.8	29.2	23.8	Positive	
	9	30.1	29.4	28.6	23.8	Positive	
	10	30.1	29.4	29.1	24.0	Positive	
	11	29.8	29.5	29.4	24.3	Positive	
	12	30.1	29.7	29.1	24.6	Positive	
	13	30.7	30.1	28.4	25.1	Positive	

Table 27 LoD determination in BAL *(continued)*

Effective Concentration	Replicate	Mean C_t				Interpretation	% Positive
		ORF1ab	N	S	MS2		
10 GCE/reaction	14	30.4	29.8	29.1	24.8	Positive	100%
	15	30.2	29.8	29.7	24.9	Positive	
	16	30.3	29.8	29.4	24.6	Positive	
	17	30.4	30.0	31.5	24.7	Positive	
	18	30.4	30.1	29.3	24.9	Positive	
	19	30.9	29.7	29.2	25.4	Positive	
	20	30.3	29.9	29.4	25.7	Positive	

Table 28 LoD determination in Nasopharyngeal specimens

Effective Concentration	Replicate	Mean C_t				Interpretation	% Positive
		ORF1ab	N	S	MS2		
10 GCE/reaction	1	30.0	28.9	35.7	25.7	Positive	100%
	2	30.6	28.9	33.6	25.8	Positive	
	3	30.2	28.8	32.0	25.8	Positive	
	4	30.4	28.7	34.2	25.7	Positive	
	5	30.5	29.0	31.4	25.8	Positive	
	6	31.0	29.3	36.6	26.0	Positive	
	7	30.3	29.2	31.1	25.8	Positive	
	8	31.1	29.2	31.8	26.5	Positive	
	9	30.5	28.9	33.0	26.2	Positive	
	10	30.3	28.8	34.7	26.8	Positive	
	11	30.5	29.8	38.7	27.4	Positive	
	12	31.6	29.7	35.0	27.6	Positive	
	13	30.7	29.3	36.4	27.4	Positive	
	14	31.6	28.8	31.3	27.2	Positive	
	15	31.0	29.3	36.0	27.0	Positive	
	16	30.5	29.1	35.7	27.0	Positive	
	17	30.7	29.4	34.8	27.4	Positive	



Table 28 LoD determination in Nasopharyngeal specimens *(continued)*

Effective Concentration	Replicate	Mean C_t				Interpretation	% Positive
		ORF1ab	N	S	MS2		
10 GCE/reaction	18	30.7	29.3	34.6	27.5	Positive	100%
	19	31.0	29.3	35.9	28.7	Positive	
	20	30.4	29.2	32.7	28.4	Positive	

Table 29 LoD results

Specimen type	Limit of Detection (GCE/reaction)
Bronchoalveolar lavage	10 GCE/reaction
Nasopharyngeal swab	10 GCE/reaction

Limit of detection (LoD) for RT-PCR with 17.5 µL and 14.0 µL of purified sample RNA

The LoD study with 17.5 µL and 14.0 µL of purified sample RNA established the lowest SARS-CoV-2 viral concentration (Genomic Copy Equivalents or GCE) that can be detected by the TaqPath™ COVID-19 Combo Kit Advanced at least 95% of the time. Banked Nasopharyngeal swab (NP) samples were pooled and spiked with gamma-irradiated SARS-CoV-2 virus at several concentrations and processed through the TaqPath™ COVID-19 Combo Kit Advanced workflow. A three-phase approach was used to determine the LoD for each specimen type. In phases I and II, the preliminary LoD was established and confirmed in phase III by testing 20 replicates.

Table 30 7500 Fast Dx Real-Time PCR Instrument (96-well), 17.5 µL of purified sample RNA

Concentration	Replicate	ORF1ab	N gene	S gene	MS2	Interpretation	% Positive
50 GCE/mL (≤7 GCE/ reaction)	1	32.975	33.685	32.953	22.847	Positive	100%
	2	33.166	33.585	35.330	22.603	Positive	
	3	33.935	33.298	33.235	22.608	Positive	
	4	33.950	33.259	33.290	22.646	Positive	
	5	35.133	Undetectable	33.382	22.605	Positive	
	6	32.808	34.349	36.963	22.422	Positive	
	7	33.917	33.736	33.330	22.164	Positive	
	8	34.014	33.378	33.422	22.589	Positive	
	9	34.266	37.113	34.356	22.373	Positive	
	10	35.234	33.341	33.372	22.726	Positive	



Table 30 7500 Fast Dx Real-Time PCR Instrument (96-well), 17.5 µL of purified sample RNA *(continued)*

Concentration	Replicate	ORF1ab	N gene	S gene	MS2	Interpretation	% Positive
50 GCE/mL (≤7 GCE/ reaction)	11	35.012	35.053	35.610	23.059	Positive	100%
	12	34.519	37.033	34.424	22.762	Positive	
	13	35.234	35.736	33.564	22.623	Positive	
	14	33.594	36.446	34.853	22.715	Positive	
	15	34.320	38.100	32.873	22.368	Positive	
	16	34.195	36.707	33.931	22.004	Positive	
	17	34.484	34.133	36.220	21.957	Positive	
	18	33.904	33.656	33.643	22.414	Positive	
	19	36.505	Undetectable	36.198	22.422	Positive	
	20	35.304	33.726	35.437	22.743	Positive	

Table 31 QuantStudio™ 7 Flex Real-Time PCR Instrument (384-well), 14.0 µL of purified sample RNA

Concentration	Replicate	ORF1ab	N gene	S gene	MS2	Interpretation	% Positive
50 GCE/mL (≤5.6 GCE/ reaction)	1	33.064	33.380	34.410	22.847	Positive	100%
	2	34.727	33.488	33.894	22.465	Positive	
	3	33.364	33.584	33.596	22.569	Positive	
	4	32.872	33.518	34.151	22.291	Positive	
	5	33.359	34.923	33.267	22.099	Positive	
	6	33.379	Undetectable	35.909	22.514	Positive	
	7	33.738	33.190	36.431	21.908	Positive	
	8	33.430	34.375	33.968	21.933	Positive	
	9	34.121	35.457	34.250	21.829	Positive	
	10	32.608	34.032	34.510	21.825	Positive	
	11	32.958	Undetectable	33.301	22.152	Positive	
	12	33.760	33.723	33.910	22.473	Positive	
	13	33.657	33.837	35.406	22.137	Positive	
	14	Undetectable	34.200	33.961	21.745	Positive	
	15	33.560	35.388	33.713	22.517	Positive	
	16	34.338	34.091	34.007	21.838	Positive	

Table 31 QuantStudio 7 Flex Real-Time PCR Instrument (384-well), 14.0 µL of purified sample RNA *(continued)*

Concentration	Replicate	ORF1ab	N gene	S gene	MS2	Interpretation	% Positive
50 GCE/mL (≤5.6 GCE/ reaction)	17	33.350	34.315	34.410	21.657	Positive	100%
	18	34.025	33.251	34.292	21.584	Positive	
	19	33.277	34.955	34.468	21.541	Positive	
	20	33.562	33.137	34.488	21.551	Positive	

Reactivity (Inclusivity)

In silico analysis was updated on June 3, 2020, using 25,998 complete SARS-CoV-2 genomes in GISAID and GenBank databases. Based upon BLAST analysis, the TaqPath™ COVID-19 Combo Kit maps with 100% homology to >99.99% of known SARS-CoV-2 isolates in GISAID and 100% of known isolates in GenBank databases. Mapping was deemed successful for a given isolate if at least two of the three targets (ORF1ab, S gene, and N gene) showed 100% identity.

Interfering substances

Pooled SARS-CoV-2-negative nasopharyngeal swab and bronchoalveolar lavage specimens were spiked with purified SARS-CoV-2 viral RNA at 3X the limit of detection (30 GCE/reaction) and potential interfering substances at the concentrations listed in Table 32 on page 114. Each substance was tested with triplicate extractions. The results are presented in Table 32 on page 114.

Pooled SARS-CoV-2-negative nasopharyngeal swab and bronchoalveolar lavage specimens were spiked with potential interfering substances at the concentrations above. Each substance was tested with triplicate extractions. No false positive results were observed for any of the substances at the concentrations tested.

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Table 32 Interfering substances

Interfering substance	Final concentration in sample	Agreement with expected results			
		Positive BAL samples	Positive NP samples	Negative BAL samples	Negative NP samples
None	N/A	100%[1]	100%	100%	100%
Mucin: bovine submaxillary gland, type I-S	0.1 mg/mL	100%[2]	100%	100%	100%
Blood (human)	1% v/v	100%[3]	100%	100%	100%
Nasal sprays or drops—Nasacort™	10% v/v	100%[4]	100%[4]	100%	100%
Nasal corticosteroids—Dymista™	5 µg/mL	100%[2]	100%	100%	100%
NeilMed™ Nasogel™	1% w/v	100%[2]	100%	100%	100%
Influenza A H1N1 Brisbane/59/07	1×10^5 $TCID_{50}$/mL	100%[2]	100%	100%	100%
Throat lozenges, oral anesthetic and analgesic—Chloraseptic™	1% w/v	100%[3]	100%	100%	100%
Oseltamivir phosphate	33 µg/mL	100%[2]	100%	100%	100%
Antibiotic, nasal ointment—Bactroban™	5 µg/mL	100%[2]	100%	100%	100%
Antibacterial, systemic—Tobramycin	0.6 mg/mL	100%[2]	100%	100%	100%
Homeopathic allergy relief medicine—Similasan™ Nasal	10% v/v	100%	100%	100%	100%

[1] Two of six replicates produced a C_t >37 or Undetermined for S Gene, but all replicates were called Positive based on the interpretation algorithm.
[2] Two of three replicates produced a C_t >37 or Undetermined for S Gene, but all replicates were called Positive based on the interpretation algorithm.
[3] All three replicates produced a C_t >37 or Undetermined for S Gene but were called Positive based on the interpretation algorithm.
[4] One of three replicates produced a C_t >37 or Undetermined for S Gene, but all replicates were called Positive based on the interpretation algorithm

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Cross-reactivity

In silico analysis of the following forty-three (43) organisms was performed.

Table 33 Organisms used for *in silico* cross-reactivity analysis

Human coronavirus 229E	Rhinovirus/Enterovirus
Human coronavirus OC43	Parechovirus
Human coronavirus HKU1	*Candida albicans*
Human coronavirus NL63	*Corynebacterium diphtheriae*
SARS-coronavirus	*Legionella* (non-pneumophila)
MERS-coronavirus	*Bacillus anthracis* (Anthrax)
Adenovirus	*Moraxella catarrhalis*
Human Metapneumovirus (hMPV)	*Neisseria elongata* and *Neisseria meningitidis*
Parainfluenza 1	*Pseudomonas aeruginosa*
Parainfluenza 2	*Staphylococcus epidermidis*
Parainfluenza 3	*Streptococcus salivarius*
Parainfluenza 4	*Leptospira* sp.
Influenza A	*Chlamydophila pneumoniae*
Influenza B	*Chlamydophila psittaci*
Influenza C	*Coxiella burnetii* (Q-Fever)
Enterovirus	*Staphylococcus aureus*
Respiratory Syncytial Virus A	*Haemophilus influenzae*
Respiratory Syncytial Virus B	*Legionella pneumophila*
Bordetella pertussis	*Mycobacterium tuberculosis*
Mycoplasma pneumoniae	*Streptococcus pneumoniae*
Pneumocystis jirovecii (PJP)	*Streptococcus pyogenes*

Among the tested organisms, *Neisseria elongata* showed homology for the forward and reverse primers and probe for the N gene. The forward primer showed ≥80% homology while the reverse primer and probe showed 36% homology. The N gene reverse primer and probe show low homology, therefore the risk of the non-specific amplification is low.

Blast analysis showed ≥80% homology for one assay component (forward primer, reverse primer, or probe) for select isolates. Despite ≥80% homology of one assay component for select isolates, there is no anticipated amplification because hybridization of all three assay components are necessary to generate a signal. We also found multiple instances where different assay components had ≥80%

homology to different isolates of the same species. For example, *Bacillus anthracis* strain AFS029987 had ≥80% homology to the ORF1ab forward primer while strain MCCC 1A01412 had ≥80% homology to the ORF1ab reverse primer. Since these are two different organisms, amplification is not likely to occur. The *in silico* analysis indicates that significant amplification of non-target sequences that result in cross-reactivity or potentially interfere with detection of SARS-CoV-2 is not likely to occur.

Clinical evaluation

A clinical evaluation study was performed to evaluate the performance of the TaqPath™ COVID-19 RT-PCR Kit using nasopharyngeal swab (NP) and bronchoalveolar lavage (BAL) specimens.

A total of sixty (60) contrived positive specimens were tested:

- 30 contrived positive nasopharyngeal swab (NP) specimens
- 30 contrived positive bronchoalveolar lavage (BAL) specimens

Samples were contrived by spiking known concentrations of extracted SARS-CoV-2 viral genomic RNA, relative to the product LoD, into matrices which were determined to be negative by the TaqPath™ COVID-19 RT-PCR Kit prior to spiking in the RNA.

In addition to the contrived positive specimens, sixty (60) negative specimens were tested:

- 30 negative nasopharyngeal swab (NP) specimens
- 30 negative samples bronchoalveolar lavage (BAL) specimens
- All negative samples yielded negative results

Results for positive samples are shown in the tables below:

Table 34 BAL Clinical Evaluation Study

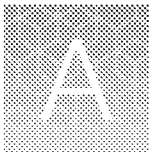
Final RNA Concentration in Sample	Number of Positives	Mean C_t		
		S gene	ORF1ab	N gene
2X LoD	20/20[1]	28.9	29.5	28.7
3X LoD	5/5[1]	28.8	29.2	28.5
5X LoD	5/5	27.4	28.2	27.4

[1] Two samples initially gave inconclusive results and were retested. The results were positive after the retest. Mean C_t values are calculated from the retest results.

Table 35 NP Clinical Evaluation Study

Final RNA Concentration in Sample	Number of Positives	Mean C_t		
		S gene	ORF1ab	N gene
2X LoD	20/20[1]	30.9	30.6	29.3
3X LoD	5/5	30.0	30.1	28.8
5X LoD	5/5	28.7	29.0	27.9

[1] One sample initially gave an inconclusive result and was retested. The result was positive after the retest. Mean C_t values are calculated from the retest results.



Example extraction plate and RT-PCR reaction plate layouts (Everlywell™ COVID-19 Test Home Collection Kit samples)

Everlywell™ COVID-19 Test Home Collection Kit samples require that users prepare two RT-PCR plate wells for each sample and Negative Control—one for the COVID-19 assay and one for the RNase P assay—plus a single RT-PCR plate well for the Positive Control. A single 96-well extraction plate contains enough samples for two 96-well RT-PCR reaction plates, or may be combined with another extraction plate onto a single 384-well RT-PCR reaction plate.

The following diagrams provide example layouts for extraction plates and RT-PCR plates, assuming that you want to fit the maximum number of samples onto each plate.

Layout of RNA extraction plate

92 RNA samples, 2 Negative Controls

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC1	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
B	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23
C	S24	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35
D	S36	S37	S38	S39	S40	S41	S42	S43	S44	S45	S46	EMPTY
E	NC2	S47	S48	S49	S50	S51	S52	S53	S54	S55	S56	S57
F	S58	S59	S60	S61	S62	S63	S64	S65	S66	S67	S68	S69
G	S70	S71	S72	S73	S74	S75	S76	S77	S78	S79	S80	S81
H	S82	S83	S84	S85	S86	S87	S88	S89	S90	S91	S92	EMPTY

Layout of 2 96-well RT-PCR plates from 1 RNA extraction plate

Each RT-PCR plate contains the following:

46 RNA samples

1 Negative Control into 2 wells (COVID-19 and RNase P assay wells)

1 Positive Control (COVID-19 assay well)

RT-PCR plate #1

		1	2	3	4	5	6	7	8	9	10	11	12
COVID-19	**A**	NC1	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
RNase P	**B**	NC1	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
COVID-19	**C**	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23
RNase P	**D**	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23
COVID-19	**E**	S24	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35
RNase P	**F**	S24	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35
COVID-19	**G**	S36	S37	S38	S39	S40	S41	S42	S43	S44	S45	S46	PC
RNase P	**H**	S36	S37	S38	S39	S40	S41	S42	S43	S44	S45	S46	EMPTY

RT-PCR plate #2

		1	2	3	4	5	6	7	8	9	10	11	12
COVID-19	**A**	NC2	S47	S48	S49	S50	S51	S52	S53	S54	S55	S56	S57
RNase P	**B**	NC2	S47	S48	S49	S50	S51	S52	S53	S54	S55	S56	S57
COVID-19	**C**	S58	S59	S60	S61	S62	S63	S64	S65	S66	S67	S68	S69
RNase P	**D**	S58	S59	S60	S61	S62	S63	S64	S65	S66	S67	S68	S69
COVID-19	**E**	S70	S71	S72	S73	S74	S75	S76	S77	S78	S79	S80	S81
RNase P	**F**	S70	S71	S72	S73	S74	S75	S76	S77	S78	S79	S80	S81
COVID-19	**G**	S82	S83	S84	S85	S86	S87	S88	S89	S90	S91	S92	PC
RNase P	**H**	S82	S83	S84	S85	S86	S87	S88	S89	S90	S91	S92	EMPTY

Figure 9 Example plate layouts for 96-well RT-PCR reactions (Everlywell™ COVID-19 Test Home Collection Kit samples)

Appendix A Example extraction plate and RT-PCR reaction plate layouts (Everlywell™ COVID-19 Test Home Collection Kit samples)
Clinical evaluation

Layout of 2 RNA extraction plates

RNA extraction plate #1
95 RNA samples, 1 Negative Control

	1	2	3	4	5	6	7	8	9	10	11	12
A	[illegible]	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
B	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23
C	S24	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35
D	S36	S37	S38	S39	S40	S41	S42	S43	S44	S45	S46	S47
E	S48	S49	S50	S51	S52	S53	S54	S55	S56	S57	S58	S59
F	S60	S61	S62	S63	S64	S65	S66	S67	S68	S69	S70	S71
G	S72	S73	S74	S75	S76	S77	S78	S79	S80	S81	S82	S83
H	S84	S85	S86	S87	S88	S89	S90	S91	S92	S93	S94	S95

RNA extraction plate #2
94 RNA samples, 1 Negative Control

	1	2	3	4	5	6	7	8	9	10	11	12
A	[illegible]	S96	S97	S98	S99	S100	S101	S102	S103	S104	S105	S106
B	S107	S108	S109	S110	S111	S112	S113	S114	S115	S116	S117	S118
C	S119	S120	S121	S122	S123	S124	S125	S126	S127	S128	S129	S130
D	S131	S132	S133	S134	S135	S136	S137	S138	S139	S140	S141	S142
E	S143	S144	S145	S146	S147	S148	S149	S150	S151	S152	S153	S154
F	S155	S156	S157	S158	S159	S160	S161	S162	S163	S164	S165	S166
G	S167	S168	S169	S170	S171	S172	S173	S174	S175	S176	S177	S178
H	S179	S180	S181	S182	S183	S184	S185	S186	S187	S188	S189	EMPTY

Layout of 1 384-well RT-PCR plate from 2 RNA extraction plates

RT-PCR plate contains the following:
189 RNA samples

2 Negative Controls into 4 wells (2 COVID-19 and 2 RNase P assay wells)

1 Positive Control (COVID-19 assay well)

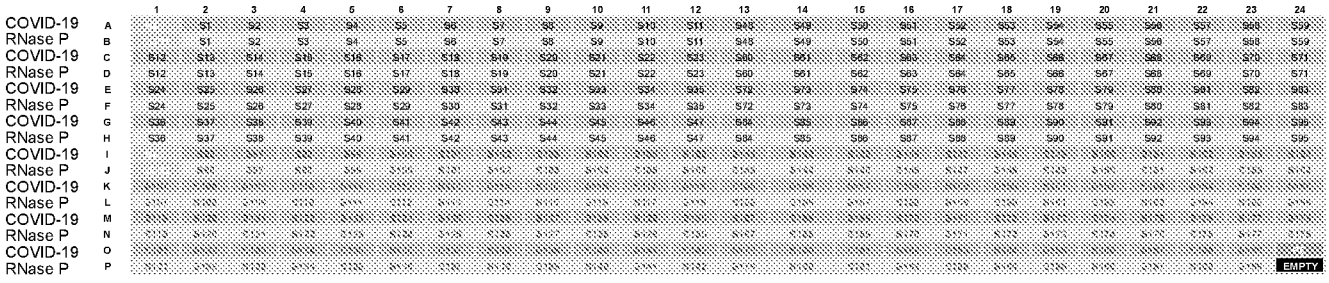
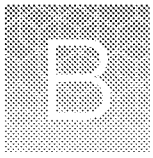


Figure 10 Example plate layouts for 384-well RT-PCR reactions (Everlywell™ COVID-19 Test Home Collection Kit samples)



C_t cutoff values for assay targets

The Applied Biosystems™ COVID-19 Interpretive Software uses the following C_t cutoff values for assay targets during interpretation of the results.

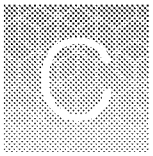
Table 36 COVID-19 Assay C_t cutoff values

Sample or Control	Target	C_t cutoff
Positive Control	MS2	Valid C_t values are >37
	Viral targets	Valid C_t values are ≤37
Negative Control	MS2	Valid C_t values are ≤32
	Viral targets	Valid C_t values are >37
Clinical samples	MS2	Valid C_t values are ≤32[1]
	Viral targets	Positive C_t values are ≤37

[1] If any of the viral targets is positive, the C_t for MS2 can be >32.

Table 37 RNase P Assay C_t cutoff values

Sample or Control	Target	C_t cutoff
Negative Control	RNase P	Valid C_t values are >33
Clinical samples	RNase P	Valid C_t values are ≤33



Documentation and support

Related documentation

Document	Publication Number
Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument Reference Guide	4406991
Applied Biosystems™ 7500/7500 Fast Real-Time PCR System: Maintenance Guide	4387777
QuantStudio™ 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide	MAN0010407
QuantStudio™ 6 and 7 Flex Real-Time PCR Systems Maintenance and Administration Guide	4489821
QuantStudio™ 6 and 7 Flex Real-Time PCR Systems Quick Reference	4489826
QuantStudio™ Real-Time PCR Software Getting Started Guide	4489822
MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (automated extraction) User Guide	MAN0018073
MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (manual extraction) User Guide	MAN0018072
Thermo Scientific™ KingFisher™ Flex User Manual	N07669
COVID-19 Interpretive Software Installation Quick Reference	MAN0019257
TaqMan™ SARS-CoV-2 RNase P Assay Kit Product Information Sheet	MAN0019524

Customer and technical support

For additional documentation and information about this kit, visit:
https://www.thermofisher.com/covid19

For download instructions for the COVID-19 Interpretive Software, see “Obtain the Applied Biosystems™ COVID-19 Interpretive Software” on page 103.

Refer to the Read Me file provided with the COVID-19 Interpretive Software before contacting support for the software.

Visit: https://www.thermofisher.com/contactus for service and support information for this kit, including the following:

- Worldwide contact telephone numbers
- Product support information
- Order and web support



- Product documentation such as:
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at www.thermofisher.com/us/en/home/global/terms-and-conditions.html. If you have any questions, please contact Life Technologies at www.thermofisher.com/support.

CLAIMS

What is claimed is:

1. A composition for amplifying three different target sequences in the SARS-CoV-2 genome, comprising:

(a) a first forward primer and a first reverse primer configured to amplify a first target sequence present in a first target region of the SARS-CoV-2 genome, wherein the first target sequence includes at least 10 contiguous nucleotides of the first target region;

(b) a second forward primer and a second reverse primer configured to amplify a second target sequence present in a second target region of the SARS-CoV-2 genome, wherein the second target sequence includes at least 10 contiguous nucleotides of the second target region; and

(c) a third forward primer and a third reverse primer configured to amplify a third target sequence present in a third target region of the SARS-CoV-2 genome, wherein the third target sequence includes at least 10 contiguous nucleotides of the third target region;

wherein the first, second and third target regions each have less than 50% similarity with an analogous region in bat-SL-CoVZC45.

2. The composition of claim 1, wherein the first target region is SEQ ID NO: 1.

3. The composition of claims 1 and 2, wherein the second target region is SEQ ID NO: 2.

4. The composition of any of the preceding claims, wherein the third target region is SEQ ID NO: 3.

5. The composition of claim 1, wherein the first target region is within the N gene of SARS-CoV-2.

6. The composition of claim 1 or 5, wherein the second target region is within the S gene of SARS-CoV-2.

7. The composition of claim 1, 5, or 6, wherein the third target region is within the ORF1ab gene of SARS-CoV-2.

8. The composition of any of the preceding claims, further including a nucleic acid sample, a polymerase, a buffer and nucleotides.

9. The composition of any of the preceding claims, further including a first probe containing a first label that is a fluorescent or other detectable label.

10. The composition of claim 9, wherein the first label of the first probe is a fluorescent label and the probe further includes a quencher that quenches the fluorescent label.

11. The composition of claims 9 or 10, further including a second probe including a second fluorescent label and a third probe including a third fluorescent label.

12. The composition of any of the preceding claims, wherein the first forward primer and the first reverse primer are selected from SEQ ID NOs: 248 and 487, or 211 and 501 or 510.

13. The composition of any of the preceding claims, wherein the second forward primer and the second reverse primer are selected from SEQ ID NOs: 5 and 441, or 100 and 337.

14. The composition of any of the preceding claims, wherein the third forward primer and the third reverse primer are selected from SEQ ID NOs: 4 and 320, or 34 and 423, or 160 and 468.

15. The composition of any of the preceding claims, further including a positive control for amplification, wherein the positive control is a synthetic plasmid comprising target regions from the SARS-CoV-2 ORF1ab gene, the S protein gene and the N protein gene.

16. A method for detecting SARS-CoV-2 in a biological sample, comprising:

(a) providing a reaction mixture containing at least a portion of the biological sample and any of the compositions of the preceding claims;

(b) subjecting the reaction mixture to amplification conditions, thereby forming one or more amplification products; and

(c) detecting at least one of the amplification products.

	Strain	Complete genome (%)	Gene region (%)												
			1ab	1a	1b	S	3	E	M	7	8	10b	N	13	14
Nucleotide sequences	Bat-SL-CoVZC45	87.6	88.9	90.7	86.0	75.2	87.8	98.7	93.4	95.2	88.8	88.5	91.1	89.1	96.7
	Bat-SL-CoVZXC21	87.5	88.7	90.3	86.1	74.7	88.9	98.7	93.4	95.2	89.1	88.5	91.2	89.5	96.7
	SARS-CoVGZ02	79.0	79.5	75.4	86.3	72.7	75.6	93.5	85.1	74.5	82.1	..	88.1	..	..
Amino acid sequences	Bat-SL-CoVZC45	..	95.6	95.6	95.8	80.2	90.9	100.0	98.6	93.4	87.6	94.2	94.3	73.2	92.9
	Bat-SL-CoVZXC21	..	95.2	95.1	95.5	79.6	92.0	100.0	98.6	93.4	88.4	94.2	94.3	73.2	92.9
	SARS-CoVGZ02	..	86.2	80.5	95.6	76.2	73.1	94.7	90.1	68.9	85.2	..	90.3	..	..

FIG. 1A

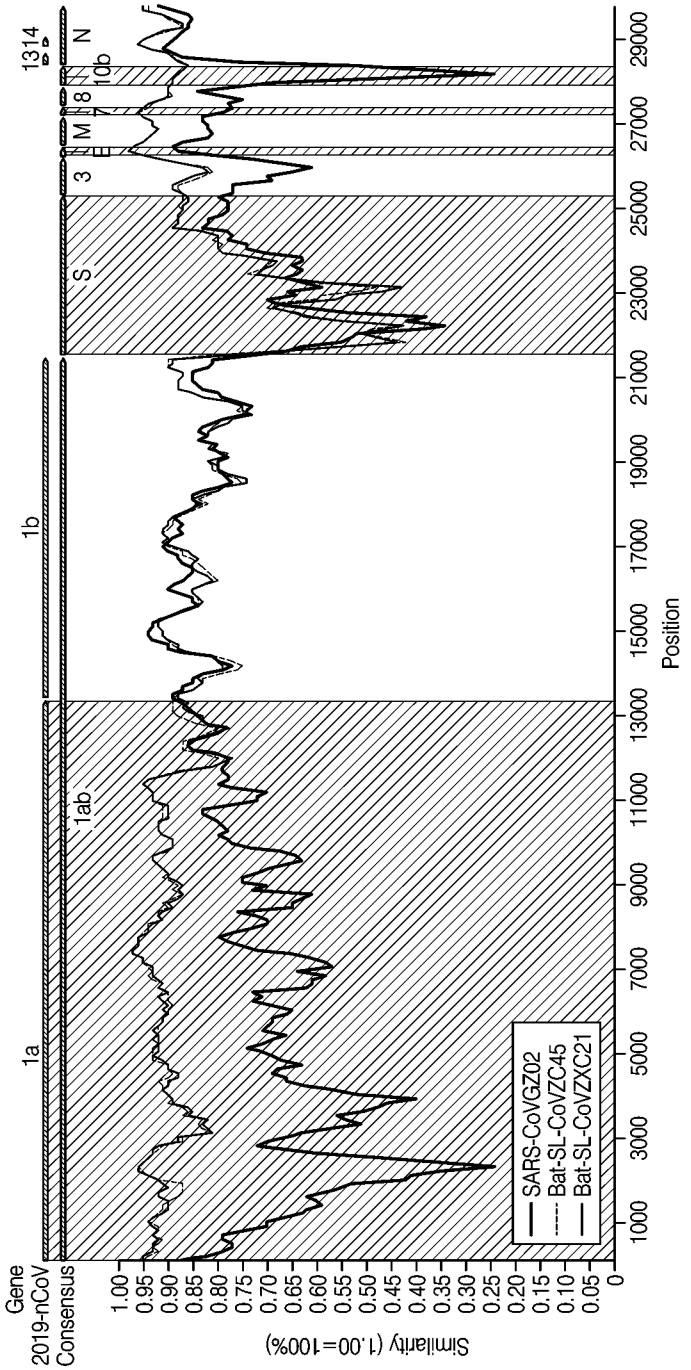

Gene
2019-nCoV
Consensus
1a
1b
1ab
S
3
E
M
8
10b
N
1314
Similarity (1.00=100%)
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
1000
3000
5000
7000
9000
11000
13000
15000
17000
19000
21000
23000
25000
27000
29000
Position
SARS-CoVGZ02
Bat-SL-CoVZC45
Bat-SL-CoVZXC21

FIG. 1B

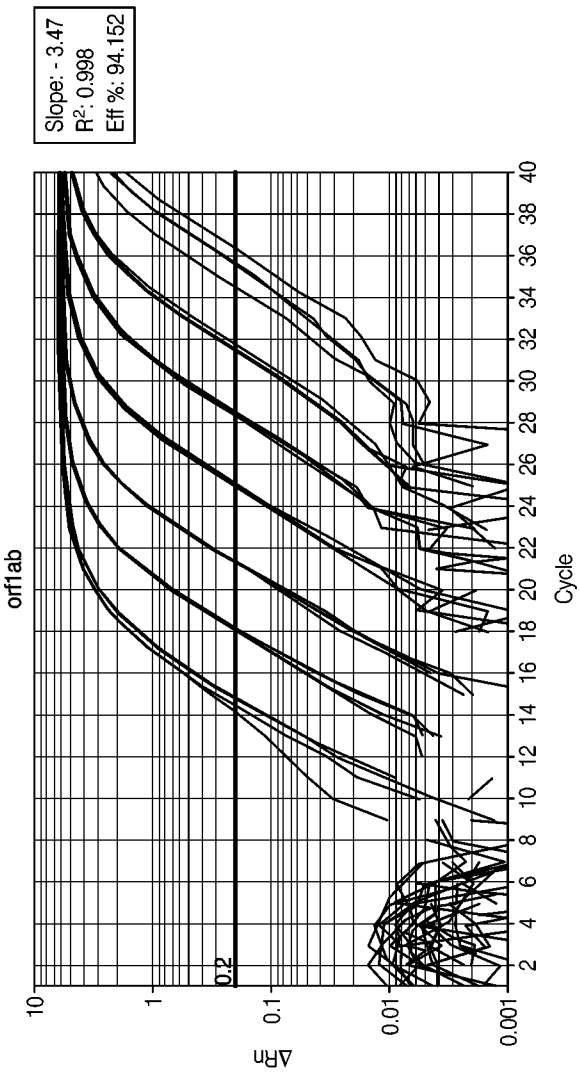

Slope: - 3.47
R2: 0.998
Eff %: 94.152
orf1ab
ΔRn
Cycle
10
1
0.1
0.01
0.001
0.2
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40

FIG. 2A

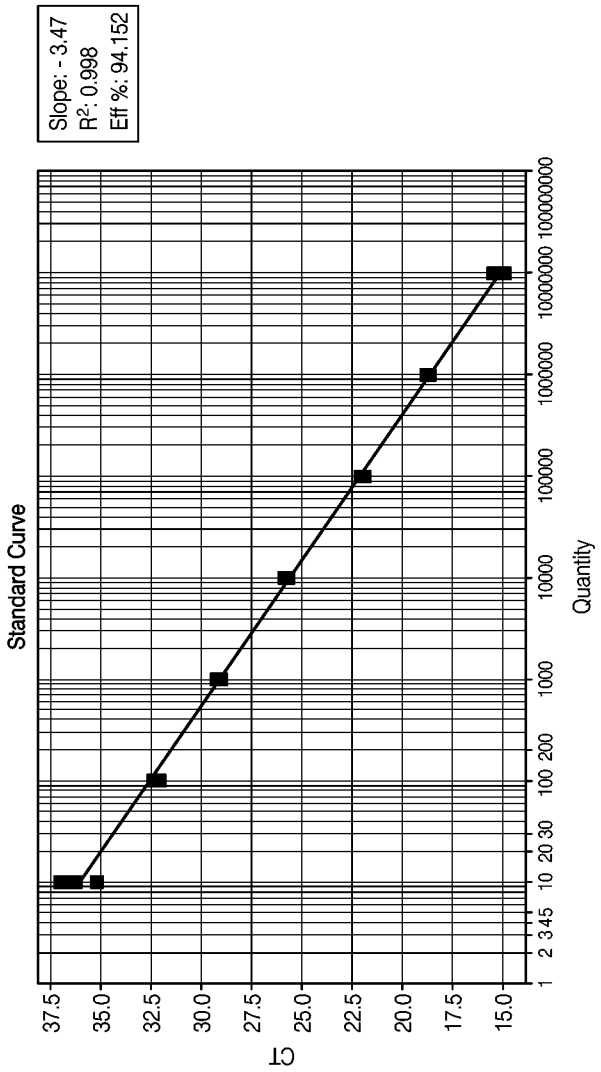

Slope: - 3.47
R2: 0.998
Eff %: 94.152
Standard Curve
Quantity
1
2
3
4
5
10
20
30
100
200
1000
10000
100000
1000000
10000000
100000000
CT
37.5
35.0
32.5
30.0
27.5
25.0
22.5
20.0
17.5
15.0

FIG. 2B

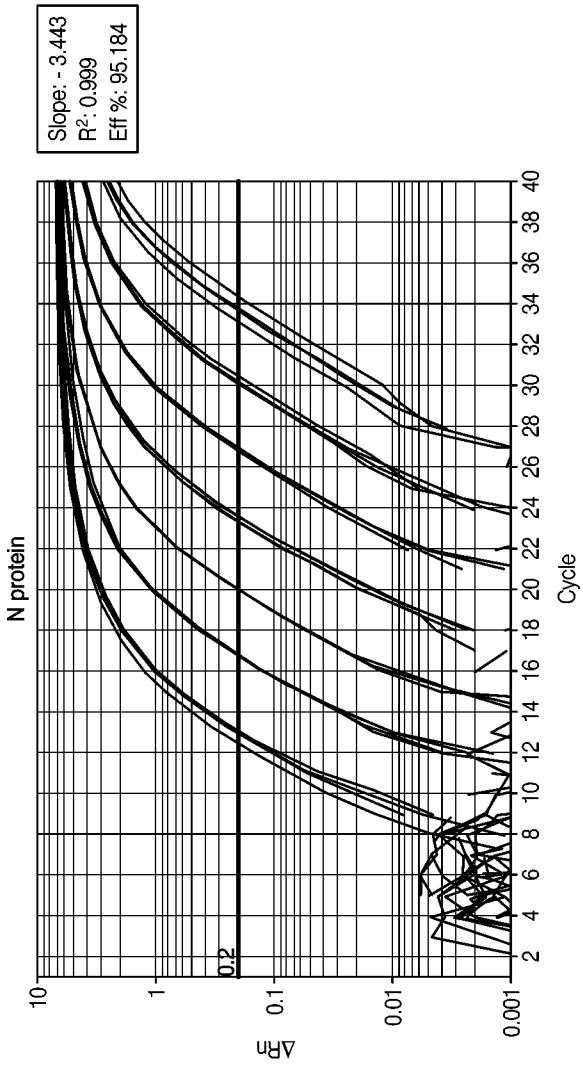

Slope: - 3.443
R2: 0.999
Eff %: 95.184
N protein
ΔRn
Cycle
10
1
0.1
0.01
0.001
0.2
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40

FIG. 2C

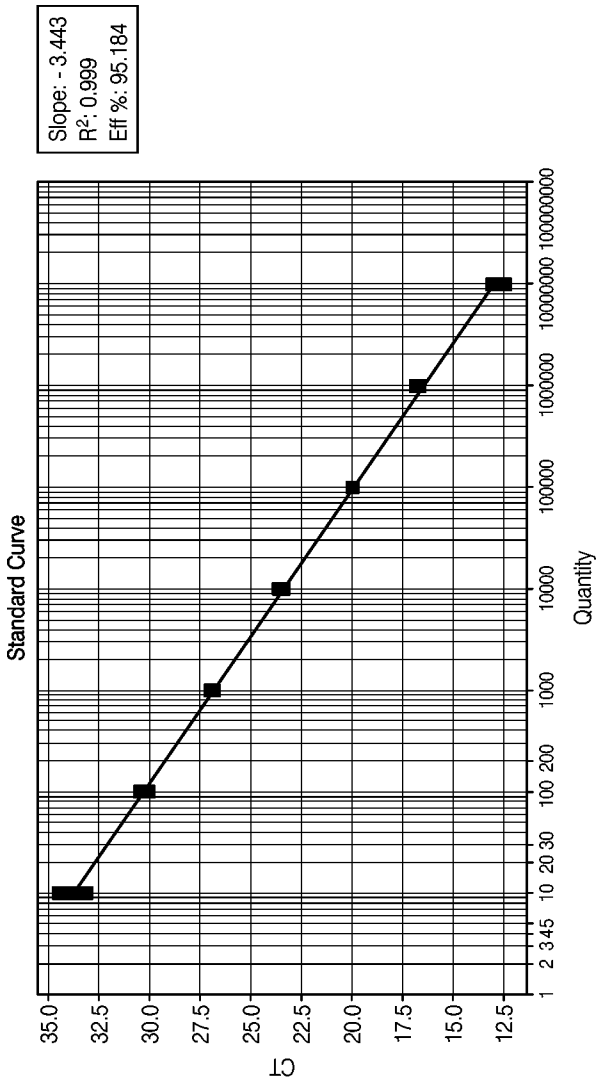

Slope: - 3.443
R²: 0.999
Eff %: 95.184
Standard Curve
CT
35.0
32.5
30.0
27.5
25.0
22.5
20.0
17.5
15.0
12.5
1
2
3
4
5
10
20
30
100
200
1000
10000
100000
1000000
10000000
100000000
Quantity

FIG. 2D

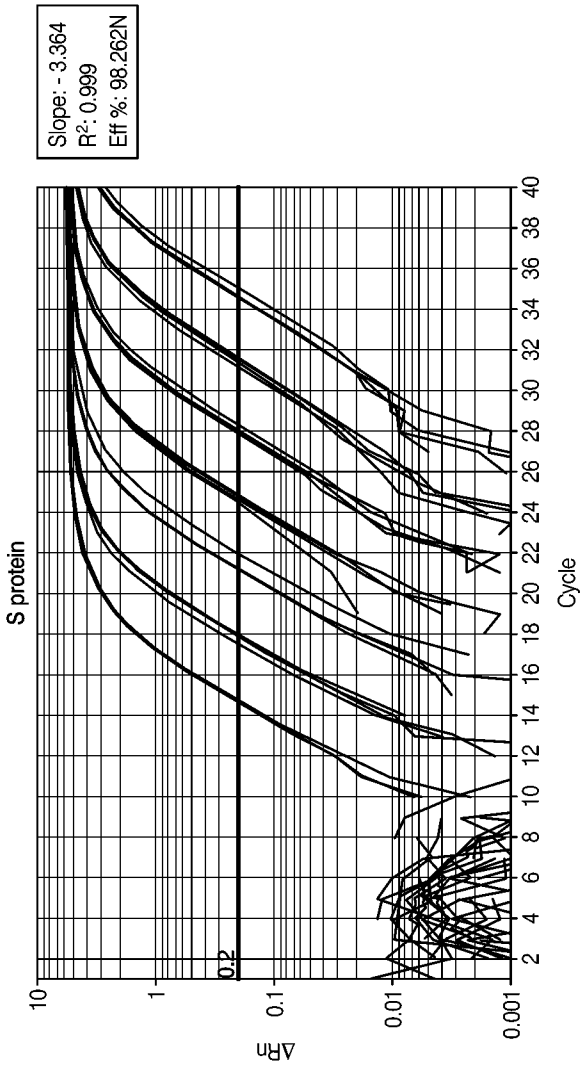

Slope: - 3.364
R2: 0.999
Eff %: 98.262N
S protein
ΔRn
Cycle
10
1
0.1
0.01
0.001
0.2
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40

FIG. 2E

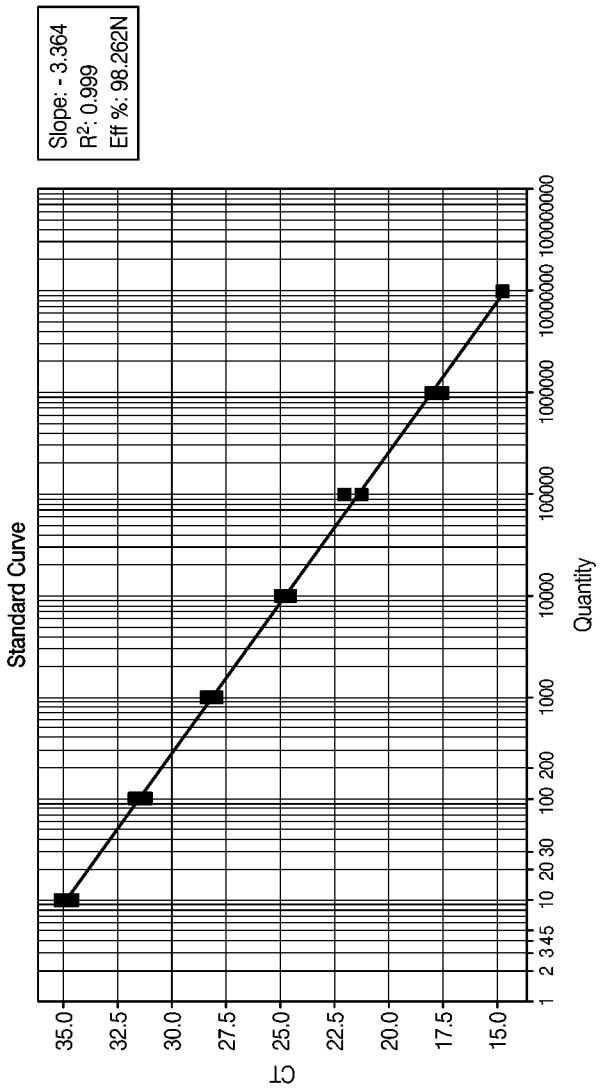

Slope: -3.364
R2: 0.999
Eff %: 98.262N
Standard Curve
CT
35.0
32.5
30.0
27.5
25.0
22.5
20.0
17.5
15.0
1
2
3
4
5
10
20
30
100
200
1000
10000
100000
1000000
10000000
100000000
Quantity

FIG. 2F

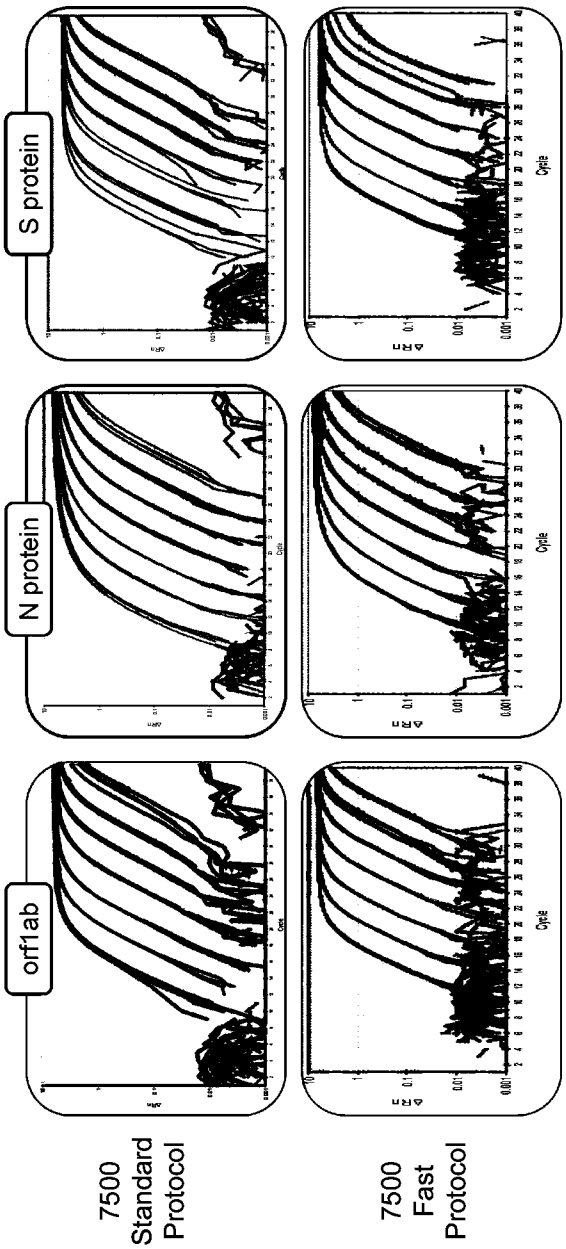


FIG. 3A FIG. 3B FIG. 3C

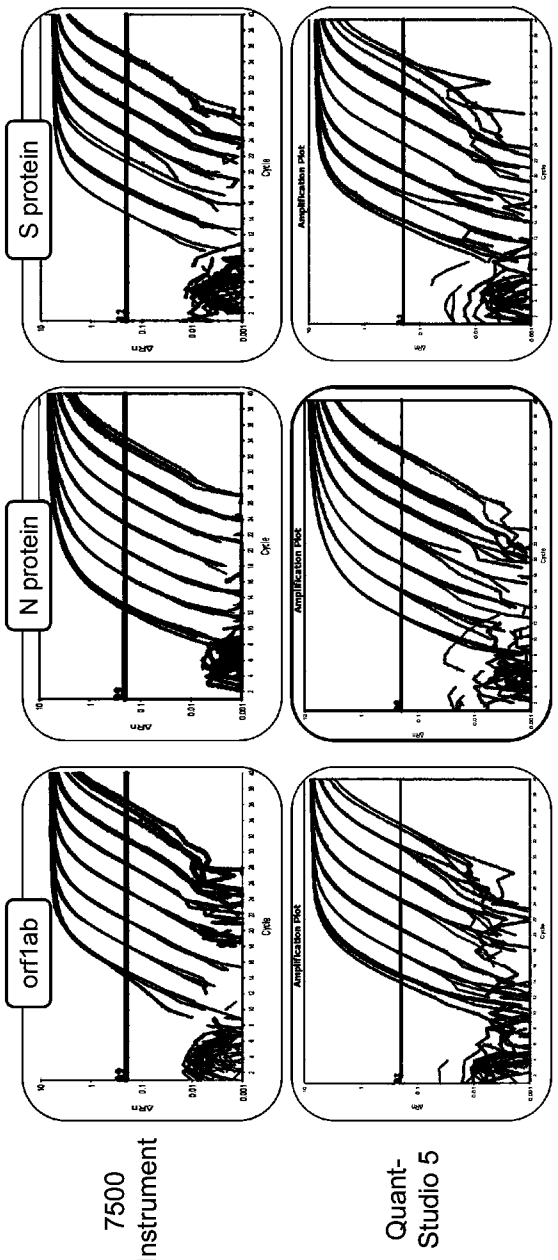


FIG. 4A FIG. 4B FIG. 4C

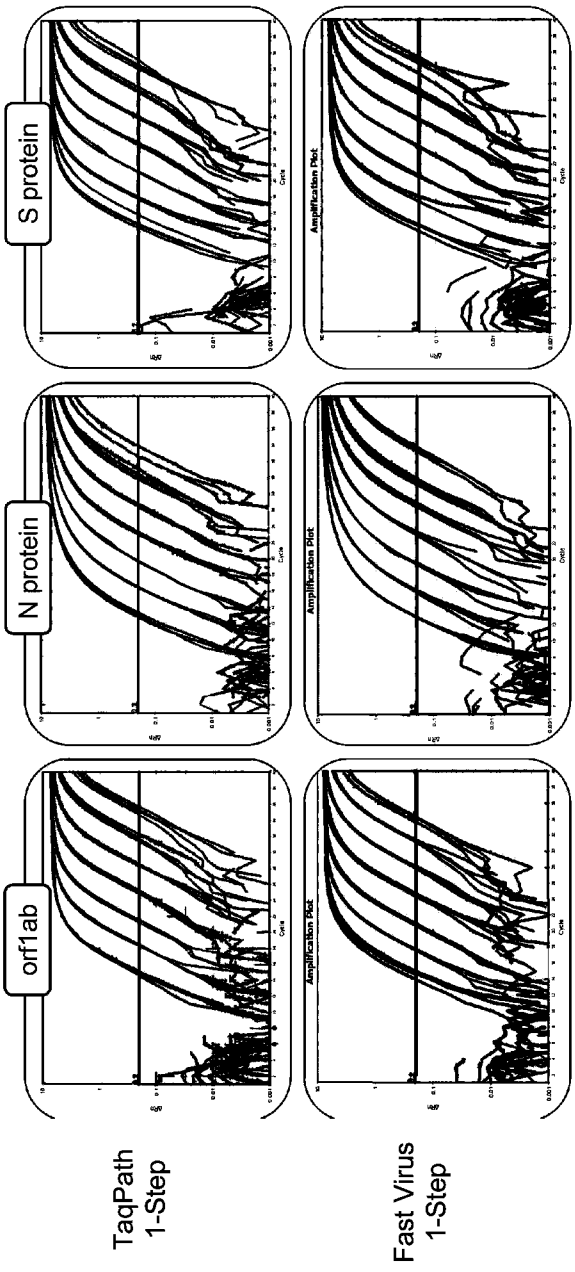


FIG. 5A FIG. 5B FIG. 5C

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2021/070163

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/70
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	VICTOR M CORMAN ET AL: "Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR", EUROSURVEILLANCE, vol. 25, no. 3, 23 January 2020 (2020-01-23), XP055695049, FR ISSN: 1560-7917, DOI: 10.2807/1560-7917.ES.2020.25.3.2000045 figures 1-2; table 1 ----- -/--	1-16

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 May 2021	09/06/2021

Name and mailing address of the ISA/	Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No
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