Multi-allelic Molecular Detection of SARS-associated Coronavirus

BACKGROUND OF THE INVENTION

Field of Invention

This invention is directed to methods for the detection and/or quantitation of the SARS virus, reagents and test kits containing the same for use in the method.

Background of the Invention

Severe acute respiratory syndrome (SARS) is one of the most recent emerging infectious diseases. The cause of SARS has been identified as a new coronavirus – a virus within the family Coronaviridae – designated as the "SARS coronavirus" (SARS-CoV) [1, 2] by the World Health Organization, following assessment of causation according to Koch's postulates, including monkey inoculation [3]. The coronaviruses are enveloped positive single-stranded RNA viruses with genomes approximately 30 kb in length – the largest of any of the RNA viruses – that replicate in the cytoplasm of host cells without going through DNA intermediates. Coronaviruses have been reported to cause common colds in humans, and to cause respiratory, enteric, and neurological diseases, as well as hepatitis, in animals. Human coronaviruses are usually difficult to culture in vitro, whereas most animal coronaviruses and SARS–CoV can easily be cultured in Vero E6 cells [4]. There are three groups of coronaviruses: Groups 1 and 2 encompass mammalian viruses, whereas Group 3 encompasses avian viruses. Within each group, the coronaviruses are classified into distinct species according to host range, antigenic relationships, and genomic organization. Human coronaviruses (HCoVs) were previously reported to belong in Group 1 (HCoV–229E) and Group 2 (HCoV–OC43), and are responsible for mild respiratory illnesses.

Recently, two independent groups, one at the British Columbia Cancer Agency (BCCA) in Canada [5] (Tor2 isolate), and the other at the Centers for Disease Control and Prevention (CDCP) in the United States [6] (Urbani isolate), were first to obtain full genomic sequences of SARS–CoV. Phylogenetic analyses, based on the genome sequences, revealed that both isolates were distantly related to previously characterized coronaviruses, including the two previously isolated nonpathogenic human coronaviruses strains, HCoV–OC43 and HCoV–229E. The genome of the Tor2 CoV isolate is 29,751 nucleotides long, and the genome of the Urbani CoV isolate is 29,727 nucleotides long, and their sequences differ at only 24 nucleotide positions. The genomic organization of both isolates is characteristic of coronaviruses having the

following typical gene order: 5'-replicase (rep), spike (S), envelope (E), membrane (M), and nucleocapsid (N). The SARS-CoV rep gene, which is approximately 20,000 nucleotides long, is predicted to encode two polyproteins (ORF1a and ORF1b) that undergo proteolytic processing, resulting in several nonstructural proteins. There are four genes downstream of rep that encode the structural proteins S, E, M, and N.

The genome of SARS–CoV has several distinct genomic characteristics that distinguish it from other coronavirus isolates and that could be of biological significance. The gene encoding hemagglutinin-esterase, which is present between ORF1a and S in Group 2 coronaviruses (and in some Group 3 coronaviruses) is absent, and so is the short anchor of the S protein. Furthermore, the short anchor of the S protein, the specific number and location of the small ORFs, and the presence of only one copy of PLP^{PRO} provide a combination of genetic features that readily distinguish SARS–CoV isolates from previously the described coronaviruses [5, 6]. There are several publications that describe reverse-transcriptase polymerase chain reaction assays (RT-PCR assays) for the detection of SARS–CoV.

Perris et al. [2] developed an RT-PCR assay that identifies the virus from nasopharyngeal aspiration samples obtained from patients infected with SARS–CoV. Total RNA from clinical samples is reverse transcribed in the presence of random hexamers, and the resulting cDNA is amplified with primers 5'-TACACACCTCAGCGTTG-3' and 5'-CACGAACGTGACGAAT-3'. To determine the genetic sequence of an unknown RNA virus, they perform a random RT-PCR assay. Total RNA from virus-infected and virus-uninfected fetal rhesus kidney cells were isolated, reverse transcribed with primer 5'-GCCGGAGCTCTGCAGAATTCNNNNNN-3', and the resulting cDNA was amplified with primer 5'-GCCGGAGCTCTGCAGAATTC-3'.

Ksiazek et al. [1] developed a reverse transcription and real-time PCR assay to identify SARS–CoV. Oligonucleotide primers used for amplification and sequencing of the SARS-related coronavirus were designed from alignments in open reading frame 1b of the coronavirus polymerase gene sequences. They used the primer pair IN-2 (+) 5'-GGGTTGGGACTATCCTAAGTGTGA-3' and IN-4 (–) 5'-TAACACACAACICCATC ATCA-3', which was previously designed to hybridize to conserved regions of open reading frame 1b (ORF1b), in order to achieve broad reactivity with the coronavirus/genus. These primers were used to amplify DNA from SARS isolates, and the amplicon sequences obtained were used to design SARS-specific primers Cor-p-F2 (+) 5'-CTAACATGCTTAGGATAATGG-

3', Cor-p-F3 (+) 5'-GCCTCTCTTGTTCTTGCTCGC-3', and Cor-p-R1 (–) 5'-CAGGTAAGCG TAAAACTCATC-3', which were used in turn to test patient specimens. Drosten et al. [4] used a PCR-based random-amplification procedure to genetically characterize a 300-nucleotide-long SARS–CoV genomic segment. On the basis of the sequence that was obtained, conventional and real-time PCR assays for specific detection SARS–CoV ORF1b were established. Poon et al. [7] developed an RT-real-time-PCR assay. Total RNA isolated from stool specimens from SARS–CoV-infected individuals is reverse transcribed with random hexamers and the resulting cDNA is amplified with primers coro3 5'-TACACACCTCAGCGTTG-3' and coro4 5'-CACGAACGTGACGAAT-3', which recognize a region of the viral polymerase gene. It is important to note that these authors acknowledged in their publication that the primers that they use in their assay can cross-react with the nonpathogenic human coronavirus strain HCoV–OC43.

SARS-specific PCR primers and diagnostic procedures were developed in several World Health Organization network laboratories for the amplification of a region of the open reading frame 1b (ORF1b) of the SARS–CoV polymerase gene sequence [8]. These primers are currently being assessed to determine their relative performance and sensitivity with different specimens obtained at different times over the course of illness. Lipkin and Briese have announced they develop a PCR-based SARS diagnostic that detects a SARS–CoV gene that is present in multiple copies, but no further information is available in the literature.

Problems with the prior art that the current invention is designed to solve. The main problems with current molecular diagnostic assays are: a) failure to consider the intrinsically polymorphic nature of coronaviruses, including the current SARS–CoV strains originated from the Tor2 and Urbani isolates – the ability of the virus to mutate and recombine during the period of time it is within the infected individual, and during horizontal transmission; and b) failure to account for the possibility of continuous and/or multiple introduction of non non-genetically identical SARS–CoV strains into the human population.

A characteristic of RNA viruses is their high rate of genetic mutation, which leads to evolution of new viral strains, and is a well-established mechanism by which viruses escape the immune system. Coronaviruses, including SARS–CoV, are quite sloppy when it comes to replicating their genetic material, producing one error for every 10,000 nucleotides that they copy, which is roughly the same error rate as occurs during the replication of the human immunodeficiency virus, HIV-1. Coronavirus RNA polymerase sometimes jumps between

multiple copies of the viral genome that are present in an infected cell. Therefore, each new genome is actually copied from several templates, reducing the chance that any given mutation will become well established in the viral population. Moreover, if one of these jumps is imprecise, a whole chunk of genome can get skipped, resulting in the deletion of part of an important gene. The consequences can be dramatic, particularly if the change affects the protein spikes that enable the virus to bind to the surface of the host's cells. For example, in 1984 a new respiratory sickness appeared on European pig farms. It turned out to be a deletion mutant of a coronavirus that previously had infected piglets' stomachs [9]. It possessed an altered spike protein that enabled the virus to infect a different cell type. Although the new disease was not generally lethal, it has since spread worldwide, and it has complicated the diagnosis of the gut disease. Another example is the recent introduction of SARS–CoV into the human population. It is likely that a genetic deletion may have helped the SARS virus to make the transition from its animal reservoir to humans. Genetics analyses of the viral strains found in animals for sale in Southern Chinese markets indicated that these SARS virus strains lack 29 nucleotides in the gene encoding a protein of unknown function, and the protein product of this gene is attached to the inside of the virus' coat protein. Furthermore, in a recent publication, full genome sequences of 14 isolates from SARS-CoV-infected patients in Singapore, Toronto, China, and Hong Kong were compared, and 14 mutations were revealed [10]. In one respect, this finding may be viewed as indicating that the SARS virus fails to mutate; however, this virus has so far encountered little resistance from its new human hosts, and there has, therefore, been little selective pressure to cause new mutants to be retained. SARS–CoV will probably not remain as stable as it has been so far. Our immune systems could force changes, similar to the changes that frequently occur in flu viruses. In summary, we deemed it prudent to develop a new SARS–CoV diagnostic assay that accounts for the genetically polymorphic nature of coronaviruses, including SARS–CoV.

SUMMARY OF THE INVENTION

The present invention includes a molecular-beacon-based multi-allelic RT-real-time-PCR assay for the detection of and discrimination between SARS-associated and other coronavirus isolates in clinical samples. The main elements of the assay design are: a) mismatch-tolerant molecular beacons; b) four sets of PCR primers for four different viral genes, and four different molecular beacons (each labeled with the same fluorophore, and each specific for a different SARS–CoV gene); c) an exogenous RNA standard that is added to the sample that can be reverse-transcribed and amplified by one of the primer sets; and d) a fifth molecular beacon that is labeled with a different fluorophore that is specific for the exogenous RNA standard. The assay further includes RNA isolation from clinical samples (blood, tissue, sputum, nasopharyngeal aspiration samples, and others), reverse transcription, PCR amplification and simultaneous automated amplicon detection in a spectrofluorometric thermal cycler that measures the fluorescence intensity of each color during the annealing phase of each thermal cycle.

Multiple target sequences within the SARS–CoV S, E, M and N genes (Urbani and Tor2 strains) were identified. The S, M, and E genes encode structural proteins that are present on the outside of the virus, whereas the N gene encodes a structural protein that is required for viral RNA packaging inside the virion. The principle underlying the selection of four target sequences that uniquely identify SARS–CoV (rather than only one target sequence) is that the use of four different targets enhances the likelihood that the fundamental genetic drift of the virus will not lead to a false negative result – that is, one has better chance of hitting a moving target with a shotgun than with a rifle. Thus, by detecting four different target alleles in the same assay tube, and by using a single-fluorophore detection system, the design of the assay significantly minimizes the likelihood of missing the presence of the SARS–CoV in a clinical sample due to the continuous viral evolution of the viral sequence. Moreover, by simultaneously detecting four different target sequences in the same assay tube, the intrinsic sensitivity of the assay is enhanced.

In order to identify the best target sequences within each viral gene that discriminate the SARS-Urbani and SARS-Tor2 strains from other nonpathogenic human and animal coronavirus strains, we used DNA alignments and phylogenetic analysis of available coronavirus gene sequences deposited in GenBank. DNA sequences of SARS–CoV genes were compared with those from reference viruses representing each species in the three known groups of

coronaviruses [group 1 (G1): human coronavirus 229E (HCoV–229E), af304460; porcine epidemic diarrhea virus (PEDV), af353511; transmissible gastroenteritis virus (TGEV), aj271965; canine coronavirus (CCoV), d13096; feline coronavirus (FCoV), ay204704; porcine respiratory coronavirus (PRCoV), z24675; – Group 2 (G2): bovine coronavirus (BCoV), af220295; murine hepatitis virus (MHV), af201929; human coronavirus OC43 (HCoV–OC43), m76373; porcine hemagglutinating encephalomyelitis virus (HEV), ay078417; rat coronavirus (RtCoV), af207551; and – Group 3 (G3): infectious bronchitis virus (IBV), m95169]. Sequence alignments were performed by CLUSTALW, which is a multiple sequence alignment tool that is commonly used in the bioinformatics community. It produces global multiple sequence alignments through three major phases: a) pairwise alignment, b) guide-tree construction, and c) multiple alignment. The guide tree generated by CLUSTALW is an estimate of relationships between sequences that are much like those shown by phylogenetic trees.

The criteria for selecting SARS–CoV gene-specific PCR primers were based on: a) the identification of genomic regions in SARS–CoV that, as a result of an examination of the sequence alignments, showed the highest genetic distance between SARS–CoV and other coronavirus strains; b) selection of primer sequences for amplification of the SARS–CoV targets that form primer-target hybrids whose theoretical melting temperature maximizes the ability of the primer to bind to the target even if nucleotide substitutions are present (mismatch tolerance), and yet enable all of the primers to hybridize to their targets at the same temperature in a multiplex assay (T_m approximately 60°C); and c) selection of primer sequences that enable the amplicons containing each of the four target sequences to be approximately the same (relatively short) length (approximately 100 nucleotides long).

The criteria for selecting the molecular beacon probe sequences, and their arm sequences, were based on: a) the identification of approximately 30-nucleotide-long regions in SARS–CoV (within the amplicons to be generated) that, as a result of an examination of the sequence alignments, showed the highest genetic distance between SARS–CoV and other coronavirus strains (with special emphasis on probe target sequences that encompass gaps or deletions in the SARS-CoV sequence compared to the sequence of other coronaviruses); b) selection of probe sequences that form probe-target hybrids whose theoretical melting temperature maximizes the ability of the probe to bind to the target sequence even if nucleotide substitutions are present (mismatch tolerance), and yet enable all of the probes to hybridize to their targets at the same temperature in a multiplex assay (Tm approximately 63°C); and c)

selection of arm sequences that provide the same degree of stability for the stem hybrids of all of the molecular beacon probes (stem T_m of approximately 70°C).

The assays described herein can be performed in either a heterogeneous or homogeneous format. The reagents needed for performance of the assay can be supplied in a kit format. The kit contains the detectants necessary for measuring two or more of the coronavirus genes S, E, M and N. It is recommended that the kit also contain an internal standard IPC. The reagents including the detectants can be separately packaged in individual ccontainers. The kits may also contain a substrate including reaction tubes for performing an assay for a given sample. The kit may also contain additional reagents for performing amplification reactions including PCR and also for saple pretreatment including those reagent necessary to release and /or purify the coronavirus.

When there is a need to perform two or more different assays on the same sample, most of the time in a single vessel and at about the same time, a multiplex format can be utilized. Such formats are known in the art. Multiplex assays are typically used to determine simultaneously the presence or concentratin of more than one molecule in the samle being analyzed, or alternatively, several characteristics of a single molecule.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the DNA Sequence Alignment of Coronavirus S Genes isolated from different species.

Figure 2 shows the Phylogenetic Analysis of S Gene.

Figure 3 shows molecular designs for SARS-Associated S Gene.

Figure 4 lists Molecular Designs for S Gene.

Figure 5 shows the DNA Sequence Alignment of Coronavirus E Genes isolated from different Species.

Figure 6 shows the phylogenetic analysis of E Gene.

Figure 7 shows molecular designs for SARS-Associated E Gene.

Figure 8 shows molecular designs for SARS-Associated E Gene

Figure 9 shows the DNA Sequence Alignment of Coronavirus M Genes isolate from different species.

Figure 10 shows the phylogenetic analysis of M Gene

Figure 11 shows the molecular designs for SARS-Associated M Gene.

Figure 12 lists molecular designs for M Gene.

Figure 13 shows the DNA Sequence Alignment of Coronavirus N Genes isolate from different species.

Figure 14 shows the phylogenetic analysis of N Gene.

Figure 15 shows the molecular designs for SARS-Associated N Gene.

Figure 16 list molecular designs for N Gene.

Figure 17 shows molecular designs for Internal Positive Control (IPC).

Figure 18 lists molecular designs for IPC.

Figure 19 shows Molecular Beacon Melting Curves.

Figure 20 shows Uniplex Real-time PCR Amplifications (Serial Dilutions-Dynamic Range).

DETAIL DESCRIPTION OF THE INVENTION

The general steps for the assay involve the performance of the following steps:

Step 1. An RNA standard that can be reverse-transcribed and amplified by one of the primer sets is added to each sample prior to isolation of RNA from the sample. RNA is then extracted from each clinical sample (nasopharyngeal aspirations, stool samples, or whole blood, obtained from patients suspected of being infected with SARS–CoV). RNA is then purified, using a QIAamp Viral RNA Kit (Qiagen, Inc., Valencia, CA), according to the manufacturer's instructions.

Step 2. The isolated RNA obtained from each clinical sample is then reverse transcribed with four target-specific primers (S Gene, S-RT 5'-AGGCTGTAAGAA-3'; E-Gene, E-RT 5'-TATTGCAGCAGTAC-3'; M Gene, M-RT 5'-AAGCAACGAAGTAG-3'; N Gene, N-RT 5'-GCCTTCTTTGTTAG-3'; Internal Positive Control, E-RT 5'-TATTGCAGCAGTAC-3'). Reverse transcription with SARS-CoV viral RNA from patient samples is performed by adding 20 μL viral RNA to a mixture of 0.125 μM of (each) gene-specific primer and incubating at 80°C for 5 minutes to denature secondary structures. The tubes are then immediately placed on ice for at least 2 minutes. Reverse transcription reactions consist of PCR buffer II (Applied Biosystems), 3 mM $MgCl_2$, 0.5 mM (each) dNTP, 10 mM dithiothreitol, 20 Units ribonuclease inhibitor (Roche Molecular Biochemicals, Indianapolis, IN) and 80 Units SuperScript II Ribonuclease H-reverse transcriptase (GibcoBRL) in a final volume of 40 μL. The reactions are then incubated at 42°C for 50 minutes, followed by inactivation at 70°C for 15 minutes.

Step 3. Real-time PCR amplification of SARS–CoV cDNA is performed using four primer pairs and five molecular beacons in the same reaction, one primer pair for each viral gene (amplification of the internal positive control is enabled by one of the primer pairs that was designed to enable the amplification of one of the for the viral gene targets). Each PCR reaction consists of 20 μL of cDNA products, 1 x PCR buffer II (Applied Biosystems), 3.5 mM $MgCl_2$, 0.5 mM (each) dNTP, 0.4 μM of each primer, and 2.5 Units of AmpliTaq Gold DNA polymerase (Applied Biosystems) in a final volume of 50 μL. Fifty cycles of amplification (94°C for 15 seconds, 53°C for 30 seconds, and 72°C for 30 seconds) are performed in an 7700

Prism spectrofluorometric thermal cycler (Applied Biosystems). For quantitative measurements, duplicates of six-fold serial dilutions (10^6 to 10 copies) of RNA standards are used as quantitative controls along with the samples being tested for a given experimental run. Viral RNA copy number for each clinical sample is calculated by interpolation of the experimentally determined threshold cycle for the test specimen onto a standard regression curve obtained from the control RNA standards (the logarithm of the number of genomic copies present in the clinical sample is inversely proportional to the observed threshold cycle).

Explanation of how the invention solves the problems of the prior art.

The main problem with the prior art is its failure to consider the intrinsically polymorphic nature of coronaviruses and to account for the possibility of continuous and/or multiple introductions of non non-genetically identical SARS–CoV strains into the human population. The present invention solves these problems by using multiple genetic sequences as targets (the S, E, M, and N genes). The primary principle of using four different genetic targets to identify SARS-CoV is to evade the fundamental genetic drift of the virus. Thus, by using four molecular beacons, each specific for a different amplified SARS–CoV target, and each labeled with same colored fluorophore, the likelihood of not detecting SARS–CoV due to continuous evolutionary changes in the virus is minimized. Furthermore, the assay design includes the presence of an internal positive control RNA, the reverse transcription and PCR amplification of which generates a signal in a different color, thus assuring that if there is an unexpected problem with RNA isolation, reverse transcription, or target amplification, the absence of the control signal will indicate that a problem occurred.

Summary of the features of other SARS nucleic acid assays and description of the differences between those assays and the current invention. As outlined in the previous sections, there are five SARS nucleic acid assays: an RT-PCR assay published by Perris et al. [2], an RT-real-time-PCR assay published by Ksiazek et al. [1], a similar RT-real-time-PCR assay published by Drosten et al. [4], another RT-real-time-PCR assay published by Poon et al. [7], and a PCR-based SARS diagnostic assay developed by Lipkin and Briese – no publication is available describing this assay. All of the published assays use RNA extracted from clinical samples. In addition, all of the published assays initiate reverse transcription of SARS–CoV RNA with random primers, and all of the published assays generate cDNA with primers previously designed to enable the amplification of conserved regions of open reading frame 1b

(ORF1b), in order to achieve broad reactivity with the coronavirus genus. The present invention differs from these inventions in many different ways. The present assay uses four different targets instead of one and it has an internal positive RNA control (artificial RNA molecule) that can be reverse-transcribed and amplified with primers designed for the viral genes (it possesses a unique target recognition sequence for detection by a unique molecular beacon). This RNA molecule serves as a control for RNA isolation, reverse transcription, and PCR amplification. The present uses real-time PCR for nucleic acid amplification and molecular beacons for real-time detection. The present employs a dual-color detection scheme, a yellow signal (tetrachlorofluorescein) for all four SARS–CoV targets and a green signal (fluorescein) indicating that the internal positive control has been isolated, reverse transcribed, and PCR amplified.

Another important aspect of our invention, which is not an aspect of the published assays, is the construction of four viral RNA targets that contain gene-specific reverse transcription sequences built in to their 3' ends. Collectively, these molecules serve as SARS-specific positive controls. All of the present designs are thermodynamically compatible to work together in a five-amplicon multiplex assay.

Also multiplex polymerase chain reaction (mPCR) are envisioned. It is a procedure for simultaneously performing PCR on greater than two different sequences. A mPCR reaction comprises: treating said extracted DNA to form single stranded complementary strands, adding a plurality of labelled paired oligonucleotide primers, each paired primer specific for a different short tandem repeat sequence, one primer of each pair substantially complementary to a part of the sequence in the sense strand and the other primer of each pair substantially complementary to a different part of the same sequence in the complementary antisense strand, annealing the plurality of paird primers to their complementary sequences, simultaneously extending said plurality of annealed primers from the 3' terminus of each primer to synthesize an extension product complementary to the strands annealed to each primer, said extension products, after separation from their complement, serving as templates for the synthesis of an extension product for the other primer of each pair, separating said extension products from said templates to produce single stranded molecules, amplifying said single stranded molecules by repeating at least once said annealing, extending and separating steps.

Lower stringent conditions are routinely used to accommodate the capture of multiple target sequences that contain variations in their nucleic acid sequences. The stringency is

reduced by either powering the temperature of hybridization and wash or by modification of the buffer. When the stringent conditions are reduced and the target nucleic acid sequence is very similar to nucleic acid sequences of another genus specificity of the capture probe for the target genus can be lost.

When multiple capture probes are used and are selected to compatible to variations in the target nucleic acid sequences, the specificity under high stringent conditions can be regained. The blending of multiple probes permits a single positive response for the presence of a grou pf target organisms.

In a multiplex assay, numberous conditions of interest are simultaneously examined. Multiplex analysis relies on the ability to sort sample components or the data associated therewith, during or after the assay is completed.

EXAMPLES

EXAMPLE 1-Design of SARS–CoV-specific molecular beacons, and primers for reverse transcription and for PCR

Purpose: The overall rationale in the design of molecular beacons and oligonuclotides for our SARS assay is to construct mismatch-tolerant molecular beacons that are thermodynamically compatible to work in a five-amplicon multiplex assay.

Design: The molecular beacons were designed so that they are able to hybridize to their targets at the annealing temperature of the PCR, while unbound molecular beacons remain in the closed conformation. These basic aspects were achieved by using coronavirus gene-specific multiple alignments and thermodynamic considerations to select the target sequences, the identity and length of the PCR primers, the identity and length of the probe sequences (target recognition sequences), and the length of the arm sequences.

Materials: In order to theoretically calculate the melting temperatures of the PCR primers and the probe-target hybrids, was used the Oligo Toolkit that is available on the internet. The melting temperatures and secondary structure predictions of the molecular beacons were

calculated by using the DNA folding program developed by Michael Zuker that is available on the internet.

Results: The theoretical melting temperatures of the PCR primers was about 60°C; the T_m of the reverse transcription primers was 47°C; the T_m of the probe- target hybrid was about 63°C; and the T_m of the stem hybrids of the molecular beacons was about 70°C.

Conclusion: The mismatch-tolerant molecular beacons that are thermodynamically compatible are designed to work in a final five-amplicon multiplex SARS–CoV assay.

See the following Figures: The molecular designs for the S gene target of the SARS–CoV assay are described in detail in Figures 1-4; for the E gene target see Figures 5-8; for the M gene target, see Figures 9-12; for the N gene target, see Figures 13-16; and for the Internal Positive Control (IPC), see Figures 17 and 18.

Example 2- Experimental characterization of the molecular beacons and the molecular beacon-target complexes

Purpose: The overall rationale of these experiments is to evaluate the thermodynamic properties of the constructed molecular beacons prior to carrying out real-time PCR experiments.

Design: For each molecular beacon, we have determined two melting curves – one for beacon alone, and one for the beacon-target complex – by using the ABI Prism 7700 spectrofluorometric thermal cycler.

Materials: For each molecular beacon, melting curves were obtained by preparing two tubes containing 50 μL of 200 nM molecular beacon dissolved in 3.5 mM $MgCl_2$ and 10 mM Tris-HCl, pH 8.0, and by adding a complementary oligonucleotide target to one of the tubes at a final concentration of 400 nM.

The fluorescence of each solution was determined as a function of temperature, using a thermal cycler with the capacity to monitor fluorescence. Temperature was decreased linearly with time from 80°C to 10°C in 1°C steps; with each holding period lasting one minute, and fluorescence intensity was measured during each hold.

Results: The theoretical melting temperature of the PCR primers was 60°C ± 2°C; the theoretical T_m of the reverse transcription primers was 47 ± 2°C; the theoretical T_m of the probe target-hybrids was about 63 ± 3°C; and the theoretical T_m of the stem hybrid of the molecular beacons was 70 ± 2°C.

Conclusion: The designed mismatch-tolerant molecular beacons was determined to correctly recognize their DNA targets, and to be thermodynamically compatible to work together in a five-amplicon multiplex SARS–CoV assay.

Figures: The melting curves of the molecular beacons and the molecular beacon-target hybrids are shown in Figure 19.

Example 3 - Uniplex SARS–CoV viral and IPC PCR amplifications, using SYBR Green to detect the amplicaons

Purpose: The overall rationale of these experiments is to evaluate the PCR primers and PCR conditions.

Design: For each SARS–CoV gene-specific and IPC amplification, a synthetic target DNA was used. PCR reactions were performed using a spectrofluorometric thermal cycler (Cepheid).

Materials: The PCR protocols are shown in the following exhibits:

for *S* Gene:

(A) SYBR Green-based Detection of *S* Gene Amplicon (LK250) of SARS-associated CoV

Mixture	Per reaction
dH_20	15 µl
10X PCR Buffer (10X)	*2.5 µl*
$MgCl_2$ (25 mM)	4.0 µl
Plat Taq DNA Pol (5 $U\mu l^{-1}$)	0.3 µl
dNTP (25 mM)	0.3 µl
LK251 (10 pmole/µl)	0.5 µl
LK252 (10 pmole/µl)	0.5 µl
Sybr Green DNA (25X)	1.0 µl
Target DNA	1.0 µl
TOTAL	25.0 µl

Smart Cycler (Cepheid)

DNA Denaturation & Enzyme Activation

Cycles: 1		
Target Temperature (° C):	**95**	**120 sec**

Primary Amplification

Cycles: 35		
Denaturation :	**95 ° C**	**15 sec**
Annealing :	**53 ° C**	**15 sec Spectra ON**
Extension:	**72 ° C**	**15 sec**

(B) Molecular-beacon-based Detection of *S* Gene Amplicon (LK250) of SARS-associated CoV

Mixture	Per reaction
dH_20	15.75 µl
10X PCR Buffer (10X)	*2.5 µl*
$MgCl_2$ (25 mM)	4.0 µl
Plat Taq DNA Pol (5 $U\mu l^{-1}$)	0.3 µl
dNTP (25 mM)	0.3 µl
LK251 (10 pmole/µl)	0.5 µl
LK252 (10 pmole/µl)	0.5 µl
LK249 Beacon (10 pmole/µl)	0.25 µl
Target DNA	1.0 µl
TOTAL	25.0 µl

Smart Cycler (Cepheid)

DNA Denaturation & Enzyme Activation

Cycles: 1		
Target Temperature (° C):	**95**	**120 sec**

Primary Amplification

Cycles: 35		
Denaturation :	**95 ° C**	**15 sec**
Annealing :	**53 ° C**	**15 sec Spectra ON**
Extension:	**72 ° C**	**15 sec**

(C) SYBR Green-based Detection of *S* Gene Amplicon (T7- and RT-Amplicon)

Mixture		Per reaction
dH_20		15 µl
10X PCR Buffer (10X)	*2.5 µl*	
$MgCl_2$ (25 mM)		4.0 µl
Plat Taq DNA Pol (5 $U\mu l^{-1}$)		0.3 µl
dNTP (25 mM)		0.3 µl
LK251-T7 (10 pmole/µl)		0.5 µl
LK252-RT (10 pmole/µl)		0.5 µl
Sybr Green DNA (25X)		1.0 µl
Target DNA		1.0 µl
TOTAL		25.0 µl

Smart Cycler (Cepheid)

DNA Denaturation & Enzyme Activation

Cycles: 1		
Target Temperature (° C):	**95**	**120 sec**

Primary Amplification

Cycles: 35		
Denaturation :	**95 ° C**	**15 sec**
Annealing :	**53 ° C**	**15 sec Spectra ON**
Extension:	**72 ° C**	**15 sec**

for the *E* Gene:

(A) SYBR Green-based Detection of *E* Gene Amplicon (LK254) of SARS-associated CoV

Mixture	Per reaction
dH_20	15 µl
10X PCR Buffer (10X)	*2.5 µl*
$MgCl_2$ (25 mM)	4.0 µl
Plat Taq DNA Pol (5 $U\mu l^{-1}$)	0.3 µl
dNTP (25 mM)	0.3 µl
LK255 (10 pmole/µl)	0.5 µl
LK256 (10 pmole/µl)	0.5 µl
Sybr Green DNA (25X)	1.0 µl
Target DNA	1.0 µl
TOTAL	25.0 µl

Smart Cycler (Cepheid)

DNA Denaturation & Enzyme Activation

Cycles: 1		
Target Temperature (° C):	**95**	**120 sec**

Primary Amplification

Cycles: 35		
Denaturation :	**95 ° C**	**15 sec**
Annealing :	**53 ° C**	**15 sec Spectra ON**
Extension:	**72 ° C**	**15 sec**

(B) Molecular-beacon-based Detection of *E* Gene Amplicon (LK254) of SARS-associated CoV

Mixture	Per reaction
dH_20	15.75 µl
10X PCR Buffer (10X)	*2.5 µl*
$MgCl_2$ (25 mM)	4.0 µl
Plat Taq DNA Pol (5 $U\mu l^{-1}$)	0.3 µl
dNTP (25 mM)	0.3 µl
LK255 (10 pmole/µl)	0.5 µl
LK256 (10 pmole/µl)	0.5 µl
LK253 Beacon (10 pmole/µl)	0.25 µl
Target DNA	1.0 µl
TOTAL	25.0 µl

Smart Cycler (Cepheid)

DNA Denaturation & Enzyme Activation

Cycles: 1		
Target Temperature (° C):	**95**	**120 sec**

Primary Amplification

Cycles: 35		
Denaturation :	**95 ° C**	**15 sec**
Annealing :	**53 ° C**	**15 sec Spectra ON**
Extension:	**72 ° C**	**15 sec**

(C) SYBR Green-based Detection of *E* Gene Amplicon (T7- and RT-Amplicon)

Mixture	Per reaction
dH_20	15 µl
10X PCR Buffer (10X)	*2.5 µl*
$MgCl_2$ (25 mM)	4.0 µl
Plat Taq DNA Pol (5 $U\mu l^{-1}$)	0.3 µl
dNTP (25 mM)	0.3 µl
LK255-T7 (10 pmole/µl)	0.5 µl
LK256-RT (10 pmole/µl)	0.5 µl
Sybr Green DNA (25X)	1.0 µl
Target DNA	1.0 µl
TOTAL	25.0 µl

Smart Cycler (Cepheid)

DNA Denaturation & Enzyme Activation

Cycles: 1		
Target Temperature (° C):	**95**	**120 sec**

Primary Amplification

Cycles: 35		
Denaturation :	**95** ° C	**15 sec**
Annealing :	**53** ° C	**15 sec Spectra ON**
Extension:	**72** ° C	**15 sec**

for the *M* Gene

(A) SYBR Green-based Detection of *M* Gene Amplicon (LK258) of SARS-associated CoV

Mixture	Per reaction
dH_20	15 µl
10X PCR Buffer (10X)	*2.5 µl*
$MgCl_2$ (25 mM)	4.0 µl
Plat Taq DNA Pol (5 $U\mu l^{-1}$)	0.3 µl
dNTP (25 mM)	0.3 µl
LK259 (10 pmole/µl)	0.5 µl
LK260 (10 pmole/µl)	0.5 µl
Sybr Green DNA (25X)	1.0 µl
Target DNA	1.0 µl
TOTAL	25.0 µl

Smart Cycler (Cepheid)

DNA Denaturation & Enzyme Activation

Cycles: 1		
Target Temperature (° C):	**95**	**120 sec**

Primary Amplification

Cycles: 35		
Denaturation :	**95 ° C**	**15 sec**
Annealing :	**53 ° C**	**15 sec Spectra ON**
Extension:	**72 ° C**	**15 sec**

(B) Molecular-beacon-based Detection of *M* Gene Amplicon (LK258) of SARS-associated CoV

Mixture	Per reaction
dH_20	15.75 µl
10X PCR Buffer (10X)	*2.5 µl*
$MgCl_2$ (25 mM)	4.0 µl
Plat Taq DNA Pol (5 $U\mu l^{-1}$)	0.3 µl
dNTP (25 mM)	0.3 µl
LK259 (10 pmole/µl)	0.5 µl
LK260 (10 pmole/µl)	0.5 µl
LK257 Beacon (10 pmole/µl)	0.25 µl
Target DNA	1.0 µl
TOTAL	25.0 µl

Smart Cycler (Cepheid)

DNA Denaturation & Enzyme Activation

Cycles: 1		
Target Temperature (° C):	**95**	**120 sec**

Primary Amplification

Cycles: 35		
Denaturation :	**95 ° C**	**15 sec**
Annealing :	**53 ° C**	**15 sec Spectra ON**
Extension:	**72 ° C**	**15 sec**

(C) SYBR Green-based Detection of *M* Gene Amplicon (T7- and RT-Amplicon)

Mixture	Per reaction
dH_20	15 µl
10X PCR Buffer (10X)	*2.5 µl*
$MgCl_2$ (25 mM)	4.0 µl
Plat Taq DNA Pol (5 $U\mu l^{-1}$)	0.3 µl
dNTP (25 mM)	0.3 µl
LK259-T7 (10 pmole/µl)	0.5 µl
LK260-RT (10 pmole/µl)	0.5 µl
Sybr Green DNA (25X)	1.0 µl
Target DNA	1.0 µl
TOTAL	25.0 µl

Smart Cycler (Cepheid)

DNA Denaturation & Enzyme Activation

Cycles: 1		
Target Temperature (° C):	**95**	**120 sec**

Primary Amplification

Cycles: 35		
Denaturation :	**95 ° C**	**15 sec**
Annealing :	**53 ° C**	**15 sec Spectra ON**
Extension:	**72 ° C**	**15 sec**

for the *N* Gene:

(A) SYBR Green-based Detection of *N* Gene Amplicon (LK262) of SARS-associated CoV

Mixture	Per reaction
dH_20	15 µl
10X PCR Buffer (10X)	*2.5 µl*
$MgCl_2$ (25 mM)	4.0 µl
Plat Taq DNA Pol (5 $U\mu l^{-1}$)	0.3 µl
dNTP (25 mM)	0.3 µl
LK263 (10 pmole/µl)	0.5 µl
LK264 (10 pmole/µl)	0.5 µl
Sybr Green DNA (25X)	1.0 µl
Target DNA	1.0 µl
TOTAL	25.0 µl

Smart Cycler (Cepheid)

DNA Denaturation & Enzyme Activation

Cycles: 1		
Target Temperature (° C):	**95**	**120 sec**

Primary Amplification

Cycles: 35		
Denaturation :	**95 ° C**	**15 sec**
Annealing :	**53 ° C**	**15 sec Spectra ON**
Extension:	**72 ° C**	**15 sec**

(B) Molecular-beacon-based Detection of *N* Gene Amplicon (LK262) of SARS-associated CoV

Mixture	Per reaction
dH_20	15.75 µl
10X PCR Buffer (10X)	*2.5 µl*
$MgCl_2$ (25 mM)	4.0 µl
Plat Taq DNA Pol (5 $U\mu l^{-1}$)	0.3 µl
dNTP (25 mM)	0.3 µl
LK263 (10 pmole/µl)	0.5 µl
LK264 (10 pmole/µl)	0.5 µl
LK261 Beacon (10 pmole/µl)	0.25 µl
Target DNA	1.0 µl
TOTAL	25.0 µl

Smart Cycler (Cepheid)

DNA Denaturation & Enzyme Activation

Cycles: 1		
Target Temperature (° C):	**95**	**120 sec**

Primary Amplification

Cycles: 35		
Denaturation :	**95 ° C**	**15 sec**
Annealing :	**53 ° C**	**15 sec Spectra ON**
Extension:	**72 ° C**	**15 sec**

(C) SYBR Green-based Detection of *N* Gene Amplicon (T7- and RT-Amplicon)

Mixture	Per reaction
dH_20	15 µl
10X PCR Buffer (10X)	*2.5 µl*
$MgCl_2$ (25 mM)	4.0 µl
Plat Taq DNA Pol (5 $U\mu l^{-1}$)	0.3 µl
dNTP (25 mM)	0.3 µl
LK263-T7 (10 pmole/µl)	0.5 µl
LK264-RT (10 pmole/µl)	0.5 µl
Sybr Green DNA (25X)	1.0 µl
Target DNA	1.0 µl
TOTAL	25.0 µl

Smart Cycler (Cepheid)

DNA Denaturation & Enzyme Activation

Cycles: 1		
Target Temperature (° C):	**95**	**120 sec**

Primary Amplification

Cycles: 35		
Denaturation :	**95 ° C**	**15 sec**
Annealing :	**53 ° C**	**15 sec Spectra ON**
Extension:	**72 ° C**	**15 sec**

and for Internal Positive Control (**IPC**)

(A) SYBR Green-based Detection of *Internal Positive Control* (LK266)

Mixture	Per reaction
dH_20	15 µl
10X PCR Buffer (10X)	*2.5 µl*
$MgCl_2$ (25 mM)	4.0 µl
Plat Taq DNA Pol (5 $U\mu l^{-1}$)	0.3 µl
dNTP (25 mM)	0.3 µl
LK251 (10 pmole/µl)	0.5 µl
LK256 (10 pmole/µl)	0.5 µl
Sybr Green DNA (25X)	1.0 µl
Target DNA	1.0 µl
TOTAL	25.0 µl

Smart Cycler (Cepheid)

DNA Denaturation & Enzyme Activation

Cycles: 1		
Target Temperature (° C):	**95**	**120 sec**

Primary Amplification

Cycles: 35		
Denaturation :	**95 ° C**	**15 sec**
Annealing :	**53 ° C**	**15 sec Spectra ON**
Extension:	**72 ° C**	**15 sec**

(B) Molecular-beacon-based Detection of *Internal Positive Control* (LK266)

Mixture	Per reaction
dH_20	15.75 µl
10X PCR Buffer (10X)	*2.5 µl*
$MgCl_2$ (25 mM)	4.0 µl
Plat Taq DNA Pol (5 $U\mu l^{-1}$)	0.3 µl
dNTP (25 mM)	0.3 µl
LK251 (10 pmole/µl)	0.5 µl
LK256 (10 pmole/µl)	0.5 µl
LK265 Beacon (10 pmole/µl)	0.25 µl
Target DNA	1.0 µl
TOTAL	25.0 µl

Smart Cycler (Cepheid)

DNA Denaturation & Enzyme Activation

Cycles: 1		
Target Temperature (° C):	**95**	**120 sec**

Primary Amplification

Cycles: 35		
Denaturation :	**95 ° C**	**15 sec**
Annealing :	**53 ° C**	**15 sec Spectra ON**
Extension:	**72 ° C**	**15 sec**

(C) SYBR Green-based Detection of *Internal Positive Control* (T7- and RT-Amplicon)

Mixture	Per reaction
dH_20	15 µl
10X PCR Buffer (10X)	*2.5 µl*
$MgCl_2$ (25 mM)	4.0 µl
Plat Taq DNA Pol (5 $U\mu l^{-1}$)	0.3 µl
dNTP (25 mM)	0.3 µl
LK251-T7 (10 pmole/µl)	0.5 µl
LK256-RT (10 pmole/µl)	0.5 µl
Sybr Green DNA (25X)	1.0 µl
Target DNA	1.0 µl
TOTAL	25.0 µl

Smart Cycler (Cepheid)

DNA Denaturation & Enzyme Activation

Cycles: 1		
Target Temperature (° C):	**95**	**120 sec**

Primary Amplification

Cycles: 35		
Denaturation :	**95 ° C**	**15 sec**
Annealing :	**53 ° C**	**15 sec Spectra ON**
Extension:	**72 ° C**	**15 sec**

Results: Real-time PCR amplification curves were consistent with the presence or absence of target DNA and the amplicons that were synthesized in the PCR reactions had the correct lengths.

Conclusion: The synthesized primers work well under uniform PCR conditions.

Example 4- Uniplex SARS–CoV viral and IPC PCR amplifications, using gene-specific molecular beacons

Purpose: The overall rationale of these experiments is to evaluate the molecular beacons, using the uniform PCR condition established in the tests with the PCR primers.

Design: For each SARS–CoV gene-specific and IPC amplification, a synthetic target DNA and molecular beacon are used. PCR reactions were performed using the spectrofluorometric thermal cycler (Cepheid). For each assay, utilized target dilutions are peformed to establish the linearity and the dynamic range of the molecular beacon-based real-time PCR assays.

Materials: The PCR protocols are shown above in Example 3 for the S Gene, E gene, M Gene, N Gene, and the Internal Positive Control (IPC).

Results: Real-time PCR amplification curves were consistent with the presence or absence of target DNA.

Conclusion: The SARS–CoV-specific and IPC-specific molecular beacons work well in under uniform PCR conditions. See Figure 20 (Exhibit 25).

Example 5- Uniplex SARS–CoV viral and IPC PCR amplifications using bacteriophage T7 RNA polymerase and reverse transcriptase target PCR primers

Purpose: The overall rationale of these experiments is for each of the five amplicons (SARS–CoV S, E, M, and N genes and IPC) to produce dsDNA molecules that contain a

bacteriophage T7 promoter target recognition sequence at their 5' ends and the gene-specific reverse transcriptase primer-binding site at their 3' ends. The synthesized amplicons are used for in vitro RNA production. The synthesized RNA molecules have the specific reverse transcriptase primer-binding site at their 3' ends.

Design: For each SARS–CoV gene-specific and IPC amplification, a synthetic target DNA and bacteriophage T7-RT-PCR primers are used. PCR reactions were performed using the spectrofluorometric thermal cycler (Cepheid), using SYBR green to detect the amplicons.

Materials: The PCR protocols are shown in Example 3.

Results: Real-time PCR amplification curves are consistent with the presence or absence of target DNA and the amplicons synthesized in the PCR reactions had the correct lengths.

Conclusion: SARS–CoV-specific bacteriophage T7-RT amplicons were generated for use as templates for in vitro RNA transcription, in order to produce the target RNAs that will be used to qualify the PCR assays. See Figure 20.

Example 6- In vitro RNA transcription of SARS–CoV- and IPC-specific RNA molecules

Purpose: The overall rationale for these experiments is to produce RNA molecules containing the specific reverse transction primer-binding site at their 3' ends.

Design: For each SARS–CoV gene-specific and IPC amplification, a T7-RT-PCR dsDNA amplicon (generated from Experiment 5) is used.

Materials: RNA transcripts corresponding to the four SARS–CoV-specific and IPC alleles were prepared by in vitro transcription of PCR products that contain the T7 RNA polymerase promoter site, by using a MEGAscript T7 kit (Ambion, Houston, TX).

Results: RNA molecules had the correct lengths, based on 8% polyacrylamide gel electrophoretic analysis of the product strands.

Conclusion: SARS–CoV-specific T7-RT amplicons were generated to be used for in vitro RNA transcription.

Example 7- Uniplex SARS–CoV-specific and IPC-specific RNA reverse transcription and molecular beacon-based real-time PCR

Purpose: The overall rationale of these experiments is to test whether the RNA molecules containing the specific reverse transcription primer-binding site at their 3' ends can be reverse transcribed and the generated cDNA can be amplified and detected by real-time PCR.

Design: For each SARS–CoV gene-specific and IPC amplification, a T7-RT-PCR dsDNA amplicon (generated from Experiment 5) is used.

Materials: Reverse transcription with viral and IPC RNA was performed by adding 20 μL of SARS–CoV-specific and IPC-specific RNA to 0.125 μM of SARS–CoV-specific and IPC-specific primers and incubated at 80°C for 5 minutes. The PCR-tubes were then immediately placed on ice for at least 2 minutes. the reverse transcriptase reactions contained PCR buffer II (Applied Biosystems), 3 mM $MgCl_2$, 0.5 mM of each dNTP (GibcoBRL), 10 mM dithiothreitol, 20 Units ribonuclease inhibitor (Roche Molecular Biochemicals) and 80 Units SuperScript II RNase H-reverse transcriptase (GibcoBRL) in a final volume of 40 μL. The reactions were incubated at 42°C for 50 minutes, followed by inactivation at 70°C for 15 minutes. Real-time PCR of cDNA, using 10 μL cDNA, 1 x PCR buffer, 3.5 mM $MgCl_2$, 0.5 mM of each dNTP, 0.4 μM of each molecular beacon, 0.4 μM of each PCR primer, and 2.5 Units AmpliTaq Gold DNA polymerase in a final volume of 50 μL. 35 cycles of amplification (94°C for 15 sec, 53°C for 30 sec, and 72°C for 30 sec) were performed in a 7700 Prism spectrofluorometric thermal cycler (Applied Biosystems).

Results: SARS–CoV-specific and IPC-specific RNA molecules can be reverse transcribed, and the generated cDNA can be amplified and detected by SARS–CoV-specific and IPC-specific molecular-beacons in real-time PCR assays.

Conclusion: SARS–CoV-specific RNA molecules can be used as controls in the final assay.

Example 8- Multiplex SARS–CoV-specific and IPC-specific RNA reverse transcription and molecular beacon-based real-time PCR

Purpose: The overall rationale of this experiment is to multiplex the uniplex SARS–CoV-specific and IPC-specific RT-PCR reactions in a multiplex RT-PCR reaction.

Design: All five RT-PCR reactions are incorporated into one.

Materials: Identical to Experiment 7, with the exception that all five RT primers, four sets of PCR primers, and five molecular beacons are used in a single RT-PCR reaction

Results: SARS–CoV-specific and IPC-specific RNA molecules can be reverse transcribed and generate cDNA, and can then be amplified and detected by SARS–CoV-specific and IPC-specific molecular beacons in a real-time PCR assay.

Conclusion: SARS–CoV-specific RNA molecules can be used as controls in a multiplex assay.

Example 9- Evaluate the complete SARS–CoV assay using clinical samples

Purpose: The overall rationale of these experiments is to evaluate the SARS–CoV assay using real samples (isolated SARS–CoV from cultures, primary patient isolates from saliva, and whole blood and stool specimens).

Design: Identical to Experiment 8.

Materials: Identical to Experiment 8.

Results: SARS–CoV assay detects SARS–CoV and discriminates between SARS–CoV and other non-pathogenic coronaviruses.

Conclusion: SARS–CoV assay detects SARS RNA extracted from cultured SARS strains and primary isolates.

Example 10-Clinical Evaluation

64 samples collected from 23 individuals who were clinically diagnosed to have SARS based on CDC, WHO and Health Canada case definitions.

The sample types included: bronchial lavage and sputnum (pre-mortem samples) as well as lung, liver, small and large bowel, and spleen tissue (post mortem).

65 samples from 15 patients served as controls. 26 were bone marrow samples sent for routing pathogen screening. 39 were post mortem organ samples from 10 patitnes who died during the outbreak but whose deathes were attributed to other causes including congestive heart failure, cerebrovascular accidents, atherosclerotic heart disease, chornic obstructive pulmonary disease, invasive Group A streptococcal infection, amiodorone pulmonary toxicity, and pulmonary fibrosis.

All samples were blindl assayed by usng the developed assay (usng all four genes: S, E, M, N) and the specificity was 100%.

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5. Marra, M.A., et al., The Genome sequence of the SARS-associated coronavirus. Science, 2003. 300(5624): p. 1399-404.
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8. Organization, W.H., http://www.who.int/csr/sars/primers/en/.
9. Pensaert, M., P. Callebaut, and J. Vergote, Isolation of a porcine respiratory, non-enteric coronavirus related to transmissible gastroenteritis. Vet Q, 1986. 8(3): p. 257-61.
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The content of each of the document identified above and cited in the specification documents is expressly incorporated herein by reference.

Claims:

1. In an assay for coronaviruses wherein the improvement comprises detectants specific for at least two genes selected from the group consisting of spike (S), envelope (E), membrane (M), and nucleocapsid (N).

2. The assay of claim 1 wherein the corona virus is a SARS virus.

3. The assay of claim 2 wherein the SARS virus is SARS–CoV.

4. The assay of claim 1 wherein the assay is a molecular-beacon-based multi-allelic RT-real-time-PCR assay.

5. The assay of claim 4 wherein the detectant is a molecular-beacon.

6. The assay of claim 5 wherein the molecular beacon is a mismatch-tolerant molecular beacons.

7. The assay of claim 1 wherein the assay is a PCR assay.

8. The assay of claim 7 wherein there are four sets of PCR primers for four different viral genes, and four different molecular beacons.

9. The assay of claim 8 wherein each molecular beacon is labeled with the same fluorophore.

10. The assay of claim 8 wherein each molecular beacon is specific for a different SARS–CoV gene.

11. The assay of claim 7 wherein the improvement further comprises an exogenous RNA standard that is added to the sample that can be reverse-transcribed and amplified by one of the primer sets.

12. The assay of claim 11 wherein the improvement further comprises the inclusion of a fifth molecular beacon that is labeled with a different fluorophore that is specific for the exogenous RNA standard.

13. The assay of claim 1 wherein the improvement further comprises RNA isolation from clinical samples

14. The assay of claim 13 wherein the sample includes blood, tissue, sputum, or nasopharyngeal aspiration samples.

15. The assay of claim 1 or 12 wherein the improvement further includes reverse transcription, PCR amplification and simultaneous automated amplicon detection in a spectrofluorometric thermal cycler that measures the fluorescence intensity of each color during the annealing phase of each thermal cycle.

16. The assay of claim 1 wherein the improvement further comprises the selection and detection of four target sequences that uniquely identify SARS–CoV.

17. The assay of claim 1 wherein the improvement further comprises including specific amplicon, primers and IPC standard along with the detectant for each gene.

18. The assay of claim 17 wherein the primers, amplicon and detectant include those having the formulas shown in figures 3, 7, 11 and 15 for each gene and an IPC standard selected from those shown in figure 17.

19. The assay of claim 17 wherein the primers, amplicon and detectant include those having the formulas shown in figures 4, 8, 12 and 16 for each gene and an IPC standard selected from those shown in figure 18.

20. The assay of claim 1 wherein the improvement further comprises a PCR multiplex format.

21. A kit for performing the assay of any one of claims 1-20.

22. The kit of claim 21 wherein the reagents are contained in individual containers.

23. The kit of claim 22 wherein the reagents are sufficient in amount to perform an assay of single sample.

24. The kit of claim 21 wherein the kit contains written instructions.

25. A reagent composition comprising one or more of the following reagent groups

S Gene

LK249 5'-FAM-CCCACGCCAGAAGGTAGATCACGAACTACACGTGGG-3'-Dubcyl

LK249.N 5'-FAM-GCCCACGCCAGAAGGTAGATCACGAACTACACGTGGGC-3'-Dubcyl

LK250

5'-CTCTATGTTTATAAGGGCTATCAACCTATAGATGTAGTTCGTGATCTA
CCTTCTGGTTTTAACACTTTGAAACCTATTTTTAAGTTGCCTCTTGG -3'

LK251 5'-CTCTATGTTTATAAGGGCTATCAACC-3'

LK251-T7 5'-TAATACGACTCACTATAGGCTCTATGTTTATAAGGGCTATCAACC-3'

LK252 5'-CCAAGAGGCAACTTAAAAATAGGTTTC- 3'

LK252-RT 5'- AGGCTGTAAGAACCAAGAGGCAACTTAAAAATAGGTTTC- 3'

S-RT 5'-AGGCTGTAAGAA-3';

E Gene

LK253 5'-FAM-CCTCCGCACGAAAGCAAGAAAAAGAAGTACGCCGGAGG-3'-Dubcyl

LK253.N 5'-FAM-GCCTCCGCACGAAAGCAAGAAAAAGAAGTACGCCGGAGGC-3'-Dubcyl

LK254 5'-CGGAAGAAACAGGTACGTTAATAGTTAATAGCGTACTTCTTTTTC
TTGCTTTCGTGGTATTCTTGCTAGTCACACTAGCCATCCTTACTGCGCTT -3'

LK255 5'- CGGAAGAAACAGGTACGTTAATAG -3'

LK255-T7 5'-TAATACGACTCACTATAGGCGGAAGAAACAGGTACGTTAATAG -3'

LK256 5'- AAGCGCAGTAAGGATGGCTA - 3'

LK256-RT5'- TATTGCAGCAGTACAAGCGCAGTAAGGATGGCTA - 3'

E-RT 5' –TATTGCAGCAGTAC-3';

M Gene

LK257 5'-FAM-CCTCCGACCCAATTAATTCTGTAGACAGCAGCCGGAGG-3'-Dubcyl

LK257.N 5'-FAM-GCCTCCGACCCAATTAATTCTGTAGACAGCAGCCGGAGGC-3'-Dubcyl

LK258

5'-CTTGTTTTCCTCTGGCTCTTGTGGCCAGTAACACTTGCTTGTTTTGTGCT TGCTGCTGTCTACAGAATTAATTGGGTGACTGGCGGGATTGCGATTGCAAT GGCTTG-3'

LK259 5'- CTTGTTTTCCTCTGGCTCTTG -3'

5'- TAATACGACTCACTATAGGCTTGTTTTCCTCTGGCTCTTG -3'

LK260 5'- CAAGCCATTGCAATCGCAATC- 3'

LK260-RT5'- AAGCAACGAAGTAGCAAGCCATTGCAATCGCAATC- 3'

M-RT 5'-AAGCAACGAAGTAG -3' ;or

IPC

LK265 5'-FAM-GCCCACGTACCATCTGGGGCTGTAGACAGCAGCCGTGGGC-3'-Dubcyl

LK266

5'-CTCTATGTTTATAAGGGCTATCAACCTATAGATGCTGCTGTCTACAGC

CCCAGATGGTAGTATTCTTGCTAGTCACACTAGCCATCCTTACTGCGCTT-3'

LK251 5'-CTCTATGTTTATAAGGGCTATCAACC-3'

LK251-T7 5'-TAATACGACTCACTATAGGCTCTATGTTTATAAGGGCTATCAACC-3'

LK256 5'- AAGCGCAGTAAGGATGGCTA - 3'

LK256-RT 5'- TATTGCAGCAGTACAAGCGCAGTAAGGATGGCTA - 3'

E-RT 5' –TATTGCAGCAGTAC-3'.

26. A kit containing the reagent composition of claim 25.