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# Rapid and Sensitive Detection of Multiple Genes From the SARS-Coronavirus Using Quantitative RT-PCR With Dual Systems

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The outbreak of severe acute respiratory syndrome (SARS) was caused by a newly identified coronavirus (SARS-CoV) in 2003. To detect early SARS-CoV infection, a one-step, real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay was developed that could simultaneously detect nucleocapsid (N), membrane (M), and spike (S) genes of SARS-CoV with the same PCR condition using either Applied Biosystems (ABI) Prism 7700 Sequence Detection System or Roche LightCycler. The sensitivity of this assay was evaluated using cell culture-derived viruses, in vitro transcribed viral RNA, and clinical specimens. The SARS-S, -M, and -N primer/probe sets described in this paper could detect one to ten copies of in vitro transcribed S, M, and N RNA per test using both the ABI and Roche assay systems. The relative sensitivities for detecting cell culture-derived SARS-CoV were 0.01, 0.01, and 0.001 PFU/test, respectively. It showed that SARS-N has comparable detection efficiencies to SARS2 and SARS3 which are primers sets designed by Centers for Disease Control and Prevention. In addition, SARS-S and SARS-M also demonstrated equivalent sensitivity to the commercially available RealArt HPA-Coronavirus reagents (Artus). The relative sensitivity of these primer/probe sets was also examined using human sera spiked viruses and clinical specimens from four confirmed SARS patients. Similar results as above were obtained. Specificity tests and sequence alignment showed that these primer/probe sets annealed perfectly to 31 isolates of SARS-CoV; and there was no cross detection with other coronaviruses and human respiratory tract-associated viruses. Therefore, not only is it compatible with the ABI and Roche systems, this multiple-gene detection assay also has the merit of being a rapid, safe, sensitive, and specific tool for accurate diagnosis of SARS-CoV infection. ***J. Med. Virol. 77:151–158, 2005.*** © 2005 Wiley-Liss, Inc.

**KEY WORDS:** SARS; SARS-CoV structural genes; Coronavirus; real-time quantitative RT-PCR; TaqMan assay

## INTRODUCTION

In March 2003, a novel coronavirus was identified in association with the severe acute respiratory syndrome (SARS) [Drosten et al., 2003; Ksiazek et al., 2003; Peiris et al., 2003a]. This new emerging virus is the causative pathogen of SARS and was named SARS-associated coronavirus (SARS-CoV) [Fouchier et al., 2003; Kuiken et al., 2003; Peiris et al., 2003b]. The genome of coronaviruses is about 30,000 nucleotides (nt), the largest found in the RNA viruses, and features their genes in the order of 5′-polymease (rep), spike (S),

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envelope (E), membrane (M), and nucleocapsid (N)-3′ with short untranslated regions at both termini [Rota et al., 2003]. The rep gene products are translated from genomic RNA, the remaining viral structural proteins (S, E, M, and N) are translated from subgenomic mRNAs that feature a 3′-coterminus and a 5′ leader sequence [Rota et al., 2003]. Therefore, the 3′-containing transcripts are the most abundant RNA species of coronaviruses [Holmes, 2001]. The genomic organization of SARS-CoV is similar to that of other coronaviruses, but phylogenetic analysis indicates that it should be a distinct new group within the genus of *Coronavirus* [Holmes, 2001; Brown and Tetro, 2003; Ruan et al., 2003; Tsui et al., 2003; Yeh et al., 2004].

In addition to SARS-CoV, there are three groups of coronaviruses; groups 1 and 2 viruses infect mammals only, while group 3 viruses restrict to avian [Holmes, 2001]. These viruses all belong to the family of Coronaviridae, and many viruses in this family may elicit respiratory and enteric diseases in humans and animals [Lai and Holmes, 2001; Ksiazek et al., 2003]. Group 1 and 2 of human coronaviruses (HCoV-229E and HCoV-OC43, respectively) are responsible for about 30% of upper respiratory tract illness [Lai and Holmes, 2001]. Besides coronaviruses, many other viruses, such as adenoviruses and influenza viruses, also induce lesions in the respiratory tract. Given the initial symptoms of SARS-CoV infection are so similar to those of the respiratory tract illness caused by other viruses, it is essential to develop diagnostic methods to distinguish the infections between them. Furthermore, many individuals or healthcare workers who suffered from SARS were exposed to SARS-affected patients with fever at the initial stage [Lee et al., 2003; Poutanen et al., 2003]. The availability of a rapid, sensitive, specific, and accurate diagnostic test to confirm SARS-CoV infection is essential to immediately alert healthcare professionals and, in turn, prevent others from coming into contact with probable SARS individuals.

According to the World Health Organization [WHO, 2003a], current diagnostic methodologies for SARS-CoV infection include molecular testing using reverse transcriptase-polymerase chain reaction (RT-PCR); antibody tests by enzyme linked immunosorbent assay (ELISA) or immunofluoresence assay (IFA); and virus isolation using Vero E6 cells. The methods using RT-PCR for detecting SARS-CoV viral RNA are safer and faster than cell culture-based assays. In addition, real-time RT-PCR assay systems employing fluorescent DNA probes in a 5′ exonuclease assay (TaqMan) have been developed for a variety of pathogens, including SARS-CoV [Lanciotti et al., 2000; Nijhuis et al., 2002; Warrilow et al., 2002; Poon et al., 2003; Emery et al., 2004]. These TaqMan detection assays offer advantages over the traditional RT-PCR techniques of increased sensitivity and specificity, decreased contamination, high throughput, and capable of providing absolute quantitation [Bustin, 2000]. However, laboratories performing SARS-CoV specific RT-PCR tests should still adopt quality-control procedures which include reconfirmation of positive specimens. WHO also suggests that amplifying a second genome region from SARS-CoV may increase the test specificity [WHO, 2003b]. For these purposes, a real-time quantitative RT-PCR (Q-PCR) assay was developed that can be run under a universal RT-PCR condition to amplify S, M, and N genes from SARS-CoV simultaneously for quick, sensitive, and specific detection of the virus. Moreover, the primer probe sets and the PCR condition to be illustrated here could be applied to both ABI Prism 7700 and Roche LightCycler systems, whichever is available in the laboratory.

## MATERIALS AND METHODS

### Viruses and Cliniclal Specimens

The SARS-CoV used in this study was isolated from the sputum sample of a SARS case at the Hoping Hospital, Taipei, during the epidemic stage-II outbreak [Yeh et al., 2004]. For virus preparation, $5 \times 10^6$ of Vero E6 cells were inoculated with SARS-CoV and the viruses were prepared on the 3rd day when cytopathogenic effect was observed. The harvested viruses were then quantified by plaque assay and serially diluted for later use [Beaty et al., 1989]. HCoV-229E (VR-740), HCoV-OC43 (VR-749), influenza A virus (VR-1520), influenza B virus (VR-1535), respiratory syncytial virus (VR-1401), and adenovirus (VR-1) were purchased from the American Type Culture Collection (Manassas, VA). Transmissible gastroenteritis virus (TGEV) was provided by Dr. Konan Peck (Academia Sinica, Taiwan). The SARS-CoV was manipulated in a laboratory certified with biosafety level-3 (BSL3). The clinical samples used in this study were collected from SARS patients at late and convalescent stage of SARS-CoV infection. The four SARS patients were seen in Tri-Serve General Hospital during the stage II epidemic in Taiwan. All were confirmed as meeting the World Health Organization's definitions of probable SARS cases, meaning they were showing typical clinical symptoms and were confirmed as having SARS-CoV infection by RT-PCR with SARS-specific primers [Drosten et al., 2003; Emery et al., 2004]. Confirmation was made by the Center for Disease Control of Taiwan.

### Plasmids and RNA

The M, N, and sub-fragment S1 sequences (nt 21443-22517, nt 26398-26940 and nt 28105-29370, respectively) of SARS-CoV were PCR amplified and cloned to pGST, pcDNA3.1, and pDrive to obtain pGST-M, pcDNA-N, and pcDNA-S1, respectively. The B*am* HI fragment of pGST-M was sub-cloned into the B*am* HI site of pGEM-3Z to produce pGEM-M. For in vitro transcription, the pcDNA-S1, pcDNA-N, and pGEM-M were linearlized with H*ind* III, X*ba* I, and N*co* I, respectively, and then transcribed using T7 (pcDNA-S1 and pcDNA-N) or SP6 (pGEM-M) RNA polymerases (T7 or SP6 transcription kit, Promega, Madison, WI). The in vitro transcribed RNA was purified using the RNeasy

mini kit (Qiagen), and was subjected to a tenfold serial dilution until the concentration was between $10^6$ and $10^0$ copies/μl in DEPC-treated water. They were used as standards for Q-PCR. Viral RNA was purified from 140 μl of inoculated Vero E6 lysates using QIAamp viral RNA kit (Qiagene, Valencia, CA), and was eluted in 60 μl elution buffer. The Hong Kong virus strain was obtained from the Center for Disease Control of Taiwan, and the viral RNA was prepared at the Institute of Preventive Medicine, National Defense Medical Center, Taiwan.

### Primer/Probe Design

The primers and TaqMan probes, SARS-S, SARS-M, and SARS-N, for the spike, membrane, and nucleocapsid, respectively, of SARS-CoV were selected using the Primer Express™ v1.5a software (Table I) (Applied Biosystems, Foster City, CA). These primer/probe sets were subjected to BLAST searching to ensure that they did not align to sequences other than SARS-CoV. These primer/probe sequences were identical to various isolates of SARS-CoV including Urbani, Tor2, CUHK, BJ04, and so on. The TaqMan probes were 5′ labeled with 6-carboxyl-fluoresin (FAM) reporter dye and 3′ labeled with the quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA). The primers were synthesized by MWG BioTech AG (Ebersberry, Germany) and purified by high-pressure liquid chromatography.

### Real-Time Quantitative RT-PCR Assays in Dual Systems

Real-time quantitative RT-PCR assays were performed using either ABI Prism 7700 Sequence Detection System (Applied Biosystems) or Roche LightCycler (Roche Diagnostics Applied Science, Mannheim, Germany). The TaqMan one-step RT-PCR master mix (Applied Biosystems) or the SuperScript III platinum one-step quantitative RT-PCR system (Invitrogen Corporation, Groningen, Netherlands) were applied to ABI Prism 7700 for Q-PCR. For Roche LightCycler, only the SuperScript III platinum was used. The TaqMan one-step RT-PCR mixture for ABI Prism 7700 contained 900 nM of PCR primers, 200 nM of probes, and 2 μl RNA were used in a final volume of 25 μl. The condition for RT-PCR was 48°C for 30 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec, and 60°C for 1 min. For SuperScript III platinum one-step quantitative RT-PCR in ABI Prism 7700 system, the reaction began with 50°C for 15 min, 95°C for 2 min, and followed by 45 cycles of 95°C for 15 sec and 60°C for 30 sec. For Roche LightCycler system, SuperScript III one-step RT-PCR was used and comprised of 10 μl of 2× reaction mix, 1 μl of 5 mM $MgSO_4$, 0.8 μl of SuperScript III RT/Platinum *Taq*Mix, primers/probes in a final concentration of 900 nM/200 nM and 2 μl RNA in 20 μl reaction mixture. The condition for the RT-PCR was 45°C for 10 min, 95°C for 3 min, followed by 50 cycles of 95°C for 10 sec and 60°C for 30 sec. The RT-PCR cycle number at which the fluorescence level increased above an inter-assay-calibrated threshold value was defined as the threshold cycle number ($C_T$) for the ABI Prism 7700. For Roche LightCycler it was the crossing point (Cp). The $C_T$ value for the "Non Template Control" (NTC) in the ABI system was 45 and the Cp value for the NTC in the LightCycler system was 50.

### Detection of SARS-CoV in Virus-Spiked Human Sera

To evaluate the sensitivity of the SARS-S, -M, -N, Q-PCR assays in human sera specimens, the SARS-CoV was spiked with human blood samples from three healthy donors as described previously [Huang et al., 2004]. These samples were used to mimic blood specimens corresponding to viremia stages of $2 \times 10^2$ to $2 \times 10^0$ virions/ml blood. These "viremia" sera were incubated at room temperature for 1 hr followed by centrifugation at 8,000 rpm for 10 min. Viral RNAs were then isolated from these specimens and analyzed with the Artus kit and SARS-S, -M, and -N TaqMan RT-PCR technique.

TABLE I. Primer/Probe Sets for SARS-CoV Real-Time Quantitative RT-PCR Assay

| Primer/probe[a] | Sequence (5′–3′) | Location | Tm(°C) |
|---|---|---|---|
| SARS-S | | | |
| Forward | TTGAATTGTGTGACAACCCTTTCTT | 21865-21889 | 60 |
| Reverse | CATCAGATATGTACTCGAAAGTGCAA | 21975-21950 | 59 |
| Probe | $^{FAM}$CATGGGTACACAGACACATACTATGATATTCGATAATGC$^{TAMRA}$ | 21905-21943 | 69 |
| SARS-M | | | |
| Forward | TGTAGGCTTGATGTGGCTTAGCTA | 26640-26663 | 59 |
| Reverse | AGAATGTTTGTTTCTGGGTTGAATG | 26735-26711 | 59 |
| Probe | $^{FAM}$TTCGTTGCTTCCTTCAGGCTGTTTGC$^{TAMRA}$ | 26665-26690 | 69 |
| SARS-N | | | |
| Forward | ACAAACATTGGCCGCAAATT | 29000-29019 | 59 |
| Reverse | CAGCCATGTTCCCGAAGGT | 29100-29082 | 59 |
| Probe | $^{FAM}$CAATTTGCTCCAAGTGCCTCTGCATTCTTT$^{TAMRA}$ | 29023-29052 | 69 |

[a]The sequences of these primers and probes were 100% identity with SARS-CoV isolates, TOR2 (AY274119), BJ02 (AY278487), BJ01 (AY278488), GD01 (AY278489), HKU-39849 (AY278491), CUHK-W1 (AY278554), Urbani (AY278741), BJ04 (AY279354), CUHK-Su10 (AY282752), Sin2500 (AY283794), Sin2677 (AY283795), Sin2679 (AY283796), Sin2748 (AY283797), Sin2774 (AY283798), Frankfurt1 (AY291315), TW1 (AY291451), SZ3 (AY304486), SZ16 (AY304488), SZ1 (AY304489), GZ43 (AY304490), GZ60 (AY304491), HKU-36871 (AY304492), GZ50 (AY304495), FRA (AY310120), TWC (AY321118), HSR1 (AY323977), ZMY1 (AY351680), UMC02 (AY357075), TWC2 (AY362698), TWC3 (AY362699).

TABLE II. Sensitivity Tests for SARS-S, SARS-M, SARS-N, Artus, SARS1 and SARS2 Real-Time Quantitative RT-PCR

| | | SARS-S[a] | | SARS-M[a] | | SARS-N[a] | | Artus[b] | | SARS1[a,c] | | SARS2[a,c] | | SARS3[a,c] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SARS-CoV (PFU/ml) | PFU/ test | Mean $C_T$ | Int | Mean $C_T$ | Int | Mean $C_T$ | Int | Mean $C_P$ | Int | Mean $C_T$ | Int | Mean $C_T$ | Int | Mean $C_T$ | Int |
| $2 \times 10^3$ | 9.3 | 25.3 | + | 25.2 | + | 23.9 | + | 28.3 | + | 24.0 | + | 22.9 | + | 23.1 | + |
| $2 \times 10^2$ | 0.93 | 28.2 | + | 27.2 | + | 25.7 | + | 29.7 | + | 26.4 | + | 24.9 | + | 26.3 | + |
| $2 \times 10^1$ | 0.1 | 32.3 | + | 31.3 | + | 31.6 | + | 33.5 | + | 31.2 | + | 29.9 | + | 29.7 | + |
| $2 \times 10^0$ | 0.01 | 36.0 | + | 35.6 | + | 35.4 | + | 36.3 | + | 35.5 | + | 33.2 | + | 33.4 | + |
| $2 \times 10^{-1}$ | 0.001 | 39.3 | ± | 41.4 | ± | 39.2 | + | 44.2 | ± | 41.3 | ± | 36.0 | + | 36.2 | + |
| $2 \times 10^{-2}$ | 0.0001 | 45 | — | 45 | — | 45 | — | 50[b] | — | 45 | — | 45 | — | 45 | — |

[a]The interpretation (Int) of TaqMan RT-PCR assay is based on the $C_T$ value, ΔRn and multi-component. The $C_T$ value $<45$, $\Delta Rn > 0$ and increased multicomponent can be interpreted as positive (+) results.
[b]The commercial RealArt HPA-Coronavirus LC RT-PCR reagents (Artus GmbH, Hamburg, Germany). Cp = 50 means in fluorimeter channel F1/F2, no signal is detected.
[c]The primer/probes set designed by CDC, Atlanta, Georgia [Emery et al., 2004].

## RESULTS

### Sensitivity of the Newly Designed Quantitative Polymerase Chain Reaction (Q-PCR) Assays for SARS-Associated Coronavirus (SARS-CoV)

A set of Q-PCR primers was designed for detecting SARS-CoV from the spike (S), membrane (M), and nucleocapsid (N) regions. They are designated as SARS-S, -M, and -N, respectively for each region (Table I). To examine the sensitivity of these primers, viral RNA that was prepared from SARS-CoV infected Vero E6 cells was used as the template. The amount of viruses obtained from serially diluted Vero E6 cells was between $2 \times 10^6$ and $2 \times 10^{-3}$ PFU/ml, and was used for RNA isolation. Using ABI Prism 7700 as a platform, S, M, and N viral RNA was detected in $2 \times 10^0$, $2 \times 10^0$, and $2 \times 10^{-1}$ PFU/ml of viruses, respectively (Table II). The corresponding sensitivities were 0.01, 0.01, and 0.001 PFU per 25 μl of reaction as described in Materials and Methods. Commercially available Q-PCR kits from Artus and CDC designed primer/probe sets (SARS1, 2, 3) were tested in parallel to our primers in order to compare their sensitivities. The primer/probe sets for detecting SARS-CoV polymerase region (Artus and SARS1) revealed similar sensitivity as the SARS-S and -M (Table II) while the SARS2, SARS3, and SARS-N primers exhibited nearly ten-fold sensitivities than the formers (Table II). These data suggest that the primer/probe sets used for detecting the SARS-CoV N region are more sensitive than that designed for S, M, and polymerase regions.

### Absolute Sensitivity of SARS-S, -M, and -N Q-PCR Assays using In Vitro Transcribed RNA Standards

To determine the absolute sensitivity of these primer/probe sets, the in vitro transcribed M, N, and sub-fragment of S transcripts were used as standards (Fig. 1). The in vitro transcribed RNA was diluted serially to yield $10^6$ to $10^0$ copies per microliter of RNAs, and was then used as a template for Q-PCR. Table III shows as low as 10 copies of sub-fragment S and M RNA, and 5 copies of N RNA could be detected efficiently using the ABI Prism 7700 machine. Similar sensitivity was also obtained using Roche LightCycler system (Table III). As soon as the RNA amount went below the levels specified, they could not be detected in either Q-PCR platforms and could produce variable positive rate (Table III).

### Specificity of SARS-S, -M, and -N Q-PCR Assays

To examine the specificity of SARS-S, -M, -N primer/probe sets, RNA from other coronaviruses and viruses that could induce clinical symptoms of the upper respiratory tract infection were tested. Table IV shows that all these primer sets could detect both SARS-CoV isolated from Taiwan and Hong Kong using the ABI Prism 7700 system. Neither group 1 or 2 coronaviruses (HCoV-229E, transmissible gastroenteritis virus, and HCoV-OC43), nor viruses such as adenovirus, influenza, and respiratory syncytial viruses could be detected using these primer/probe sets (Table IV). These results demonstrated that the SARS-S, -M, and -N primer/probe sets were highly specific for SARS-CoV and could be used as diagnostic tools for detecting SARS virus.

### Detection of SARS-CoV in Spiked Human Sera

To test the sensitivity and application of SARS-S, -M, -N primer/probe sets for human sera specimens, SARS-CoV obtained from inoculated Vero E6 cells was spiked with normal human sera from three individuals. It was then followed by RNA purification and Q-PCR assays. The Q-PCR assays using SARS-S, -M, -N primer/probe sets could easily detect SARS-CoV genome from spiked sera containing $2 \times 10^2$ PFU/ml of viruses which is equivalent to one virion per test (Table V). As expected, SARS-N exhibited the best sensitivity among the tests that used the SARS-S, -M, or Artus primer/probe sets (Table V).

### Evaluation With Clinical Specimens

The SARS-M, -N Q-PCR assays were applied to clinical specimens from four confirmed SARS patients. SARS-N could detect the presence of SARS-CoV in specimens of urine, serum and throat swabs as efficiently as the SARS2 primer/probe sets (Table VI). They

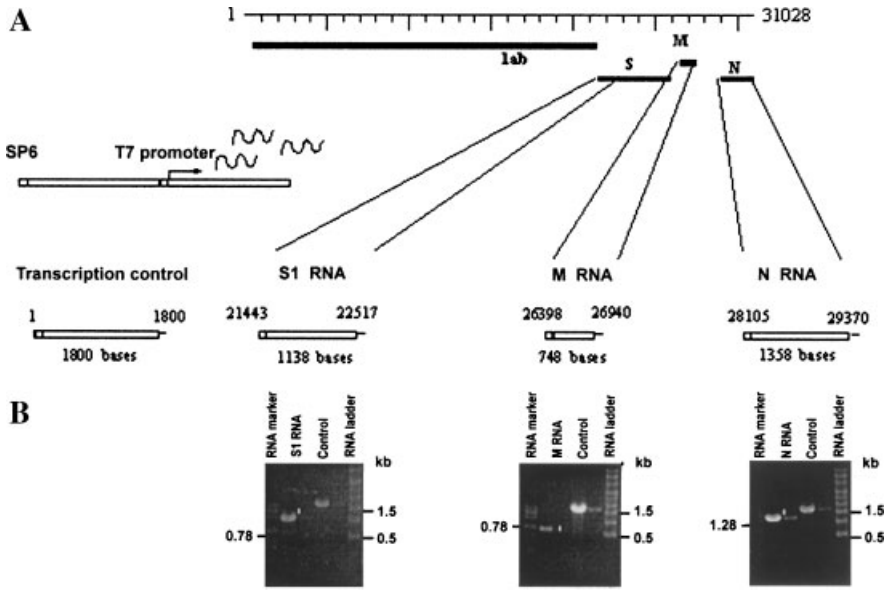


Fig. 1. **A**: Map, nucleotide positions, and sizes of in vitro transcribed SARS-S1, SARS-M, and SARS-N RNA. S1 and N RNA were transcribed using T7 RNA polymerase, M RNA was transcribed using SP6 RNA polymerase; (**B**) Denatured gel electrophoresis of these in vitro transcribed SARS subgenomic RNAs. Lane RNA marker: RNA millennium size markers (0.5–9 kb, Ambion); Lane RNA ladder: (0.16–1.77 kb, Invitrogen); Lane Control: 1,800 base RNA transcribed from control plasmid was used as a positive control.

displayed the best sensitivity with these samples among various Q-PCR assays, including the usages of the Artus and BNIOUT assays (Table VI). The sensitivity of the SARS-M primer/probe for these specimens was equivalent to the Artus and BNIOUT but was less sensitive than the SARS-N and SARS2 primer/probe sets (Table VI). All of these assays produced negative results in 32 non-SARS specimens and were specific to SARS-CoV (data not shown). These data support the tests using cell culture-derived viruses and in vitro transcribed RNA as templates; and suggest that the SARS-M, -N Q-PCR assays are suitable for clinical application.

## DISCUSSION

In this study, three primer/probe sets and a universal PCR program were designed that could detect three genes of SARS-CoV simultaneously by real-time quantitative RT-PCR. This "in-house" Q-PCR assay can use two reagent systems alternatively and can be applied to both the ABI and Roche platforms as described in Materials and Methods. If both the ABI and Roche instruments are available, it takes less than 3 hr after PCR is setup to obtain six SARS-CoV diagnostic and quantitative results (by combination of two assay platforms and three primer/probe sets). Therefore, our multi-gene quantitative RT-PCR assay provides more data for SARS diagnosis and offers the advantages of double-checking the presence of SARS-CoV RNA in the specimens as well as test specificity.

According to the guidelines suggested by WHO, positive RT-PCR results obtained from SARS specimens need to be reconfirmed by a second round of the RT-PCR test, either by repeating the same PCR test or by performing a different RT-PCR assay [WHO; WHO, 2003b]. Our multi-gene real-time RT-PCR assay is an

TABLE III. Absolute Sensitivity of SARS-S, -M, -N Real-Time Quantitative RT-PCR Assay on Dual Thermocycler Systems

| Q-PCR | SARS-S | | SARS-M | | SARS-N | |
|---|---|---|---|---|---|---|
| RNA copies | ABI mean $C_T$[a] | Roche mean $C_P$[a] | ABI mean $C_T$ | Roche mean $C_P$ | ABI mean $C_T$ | Roche mean $C_P$ |
| $10^2$ | 34.4 (3/3)[b] | 34.1 (3/3) | 35.5 (3/3) | 35.8 (3/3) | 34.0 (3/3) | 34.3 (3/3) |
| $10^1$ | 38.5 (3/3) | 37.3 (3/3) | 38.2 (3/3) | 38.9 (3/3) | 37.7 (3/3) | 37.8 (3/3) |
| $5 \times 10^0$ | 42.8 (1/3) | 38.8 (3/3) | 40.5 (2/3) | 42.1 (2/3) | 38.4 (3/3) | 38.9 (3/3) |
| $10^0$ | 45 (0/3) | 42.6 (1/3) | 43.5 (1/3) | 42.3 (1/3) | 43.4 (1/3) | 42.4 (1/3) |

[a]The $C_T$ or Cp values presented are the means of $C_T$ (Cp) from three independent experiments.
[b]Positive detection ratio of three independent experiments. The definition for positive interpretation is same as Table II.

TABLE IV. Specificity Tests of SARS-S, -M, -N Quantitative RT-PCR Assay

| Viruses[a] | Titer | | Real-time RT-PCR $C_T$ values | | |
|---|---|---|---|---|---|
| | | | SARS-S | SARS-M | SARS-N |
| SARS-CoV TW | $2 \times 10^6$ | PFU/ml | 15.34 | 14.98 | 14.88 |
| SARS-CoV HK | $10^6$ | $TCID_{50}$/ml | 24.84 | 24.42 | 22.36 |
| TGEV | $10^6$ | PFU/ml | Neg.[b] | Neg. | Neg. |
| HCoV 229E | $5 \times 10^5$ | $TCID_{50}$/ml | Neg. | Neg. | Neg. |
| HCoV OC43 | $1.7 \times 10^9$ | $LD_{50}$/ml | Neg. | Neg. | Neg. |
| Influenza A | $5 \times 10^6$ | $TCID_{50}$/ml | Neg. | Neg. | Neg. |
| Influenza B | $5 \times 10^{4.5}$ | $TCID_{50}$/ml | Neg. | Neg. | Neg. |
| RSV | $5 \times 10^5$ | $TCID_{50}$/ml | Neg. | Neg. | Neg. |
| Adenovirus | $5 \times 10^{4.5}$ | $TCID_{50}$/ml | Neg. | Neg. | Neg. |

[a]ARS-CoV TW: Taiwan isolate; SARS-CoV HK: Hong Kong isolate; TGEV: transmissible gasteroenteritis virus. RSV: respiratory syncytial virus These viral RNAs have been checked by their virus specific primer pairs as described [Sizun et al., 1998; Avellon et al., 2001; Coiras et al., 2003; Escors et al., 2003].
[b]Cp values are 50, no fluorescence signals are detected.

ideal candidate for clinical application owing to its capability of running three Q-PCR assays simultaneously plus the added benefit of satisfying the positive criteria for determining SARS-CoV infection recommended by WHO. All that is required is a single round of PCR. Such, a test that can detect multiple viral genes is of importance for preventing the spread of SARS-CoV during an outbreak because, not only can it save time from reconfirmation but also enables rapid classification and management of probable SARS cases. In addition to the ability of producing timely results, the single-step and "closed-tube" nature of this real-time RT-PCR can minimize the potential of laboratory cross-contamination as compared with the nested RT-PCR suggested by WHO.

The sensitivities of our primer/probe sets are equivalent to the commercial Artus kit and the CDC SARS1, 2, 3 primer sets when examined using cell culture-derived viruses (Table II). These assays are highly sensitive with a validated detection rate of 5–10 copies of viral RNA depending on which target gene is amplified (Table III). For the RNA containing 3′ co-terminal nucleocapsid region that is the most abundant species [Holmes, 2001], it should be the ideal amplification target and exhibits the highest detection sensitivity. Indeed, the SARS-N primer set could detect just 1 copy of viral RNA and was more sensitive than other primer sets regardless of whether the templates were obtained from cell culture-derived viruses, in vitro transcribed viral RNA, or clinical specimens (Tables II–VI). Other primer/probe sets that detect the SARS-CoV N region, such as CDC SARS2 and SARS3, also display a higher sensitivity than that detecting viral genome other than the N region (Tables II and VI). These data suggest that the primer sets for detecting SARS-CoV N region should be the best choice for high sensitive detection.

The difference between cell culture-derived viruses and in vitro transcribed viral RNA in detection sensitivity (0.01–0.001 PFU vs. 1–10 copies RNA) may be attributed to the presence of defective virions in culture supernatant. Since the genetic materials of defective virions are detectable molecularly but unable to form plaques using cell culture-based assay for quantification of virus titer [Vabret et al., 2001], the in vitro transcribed SARS-CoV RNA is more suitable for serving as the absolute quantitation standards. Furthermore, the use of these recombinant RNA can eliminate the need of dangerous virus preparation and is ideal for common laboratories.

The spiked experiments with Japanese encephalitis virus (JEV) and enterovirus showed that TaqMan Q-PCR assays worked well for a variety of spiked materials without apparent inhibition [Nijhuis et al., 2002; Huang et al., 2004]. The sensitivities of the SARS-S, -M, -N and the Artus Q-PCR assays using SARS-CoV spiked human sera were lower than those using cell culture-derived viruses. There is a difference of approximately three cycles in Q-PCR assays using these two types of templates (Table II and V). It is possible that much of the naked SARS-CoV RNA in cell culture-derived viral preparation is sensitive to certain degrading agents in human sera, such as RNase during the one-hour incubation of spiked viruses at room temperature (see Materials and Methods). However, the sensitivity of the SARS-S, -M, and -N Q-PCR assay for SARS-CoV spiked human sera is satisfied at a level that is as low as

TABLE V. Evaluation of SARS-S, -M, -N, and Artus Quantitative RT-PCR Assay Using Human Sera Spiked SARS-CoV

| Human sera | SARS-CoV PFU/ml | Real-time RT-PCR $C_T$ values | | | |
|---|---|---|---|---|---|
| | | SARS-S | SARS-M | SARS-N | Artus |
| Serum 1 | $2 \times 10^2$ | 30.8 | 29.6 | 29.5 | 29.9 |
| Serum 2 | $2 \times 10^2$ | 31.2 | 30.3 | 29.8 | 30.6 |
| Serum 3 | $2 \times 10^2$ | 31.2 | 29.7 | 28.8 | 30.1 |

TABLE VI. Results of SARS-M, -N, SARS2, Artus, and BNIOUT Quantitative RT-PCR Assays Using Clinical Specimens From Confirmed SARS Patients

| Case number | Specimens type | SARS-N[a] | SARS2[a] | SARS-M[a] | Artus[b] | BNIOUT[a] |
|---|---|---|---|---|---|---|
| 1-01 | Serum | 33.8 (+)[c] | 33.3 (+) | 45.0 (−) | 50.0 (−) | 45.0 (−) |
| | Urine | 30.7 (+) | 31.3 (+) | 37.6 (+) | 35.6 (+) | 35.0 (+) |
| 1-02 | Urine | 34.3 (+) | 34.1 (+) | 37.0 (+) | 36.7 (+) | 38.3 (+) |
| 1-04 | Throat swab | 35.3 (+) | 34.4 (+) | 35.3 (+) | 40.5 (+) | 45.0 (−) |
| | Urine | 28.3 (+) | 30.1 (+) | 30.0 (+) | 50.0 (−) | 45.0 (−) |
| 1-06 | Throat swab | 35.8 (+) | 38.5 (+) | 45.0 (−) | 50.0 (−) | 45.0 (−) |
| | Urine | 31.2 (+) | 29.2 (+) | 35.3 (+) | 50.0 (−) | 45.0 (−) |

[a]The SARS-N, SARS2, SARS-M, and BNIOUT assays were performed using ABI platform for 45 cycles.
[b]The Artus assay is performed on Roche platform for 50 cycles.
[c]Interpretation of TaqMan RT-PCR assay which is based on the criteria described in Table II.

1 PFU/test (Table V). It suggests this assay may be used in clinical specimens without apparent inhibitions.

Due to the SARS specimen control policy in Taiwan, we were unable to acquire clinical samples of SARS patients in the acute phase. The available clinical specimens were mostly obtained from the last stages of SARS-CoV infection, therefore, the viral load may be reduced in these samples as a result of the raised host immune response in late phase. This may also be the reason why the $C_T$ values from these specimens were relatively higher than those from the acute phase. However, for these specimens, our SARS-N Q-PCR assay results were as good as the CDC SARS-2 primer/probe which had been evaluated with many other human clinical samples [Emery et al., 2004]. The sensitivity of the SARS-M Q-PCR is lower than SARS-N and SARS-2 but is better than the Artus kit and BNIOUT for these clinical samples (Table VI). Based on these comparisons, our assay may be suitable for clinical diagnosis during acute phase of SARS as presented by CDC SARS2 and Artus kit.

The BLAST analysis of the primer/probe sequences and the specificity tests using several viruses demonstrated that SARS-S, -M, and -N primer/probe sets are highly specific for SARS-CoV (Tables I and IV). No cross-reactivity with other coronaviruses and the upper respiratory tract-associated viruses augment the application of these Q-PCR primer/probe sets in differentiating the infections of SARS-CoV from other viruses causing illness with similar clinical symptoms to initial stage of SARS. In addition, the SARS-S, -M, and -N primer/probe sets have been shown to distinguish clinical specimens between four confirmed SARS patients and 32 non-SARS individuals (Table VI and dada not shown). These results indicate that this Q-PCR assay is highly specific to SARS-CoV.

Besides the purpose of diagnosing SARS-CoV infection, this quantifiable RT-PCR assay can also be used as a replacement of traditional virus quantitation methods such as TCID50 or the plaque assay. Some of these traditional assays are labor-intensive, time-consuming, and more dangerous. In comparison, our assay is an ideal substitute and can be used in laboratory research for vaccine or anti-SARS drugs development. To summarize, the SARS-S, -M, and -N primer/probe sets are one-step and easy to setup assays. They are compatible with dual mainstream Q-PCR systems which means flexibility in tool selections for the operators. In addition, the assay provides accurate diagnosis for SARS-CoV-related clinical samples with cheaper homemade reagents. This is, indeed, an ideal and convenient assay for every laboratory working on SARS-CoV diagnosis, vaccine, and antiviral drug development.

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