ISSN 1021-366X

Establishment and Analysis of a SARS Real-Time RT-PCR System

Introduction

In February 2003, an American businessman developed atypical pneumonia in Hanoi, and was sent to Hong Kong for treatment, and died there. Thereafter, cases of atypical pneumonia complicated with respiration failure occurred in succession in Hong Kong and Vietnam. At the same time in the Guangdong area of the mainland China, cases of atypical pneumonia also occurred. Similar cases appeared in Taiwan in mid-March. Cases had also occurred one after another in other countries such as Canada, Singapore, and the US. For more effective distinction and definition of the infection, the World Health Organization officially named on 15 March the infection the Severe Acute Respiratory Syndrome (SARS). In April, the US CDC, by using electro-microscope and virus culture methods, successfully identified the SARS virus as a novel coronavirus. The routes of transmission of the SARS virus were speculated to be via droplets at short distance or by contact. The infection spread at an astonishing speed in many countries for its short incubation period and for the fact that many people had not developed any antibodies against it. In Taiwan, the index case was a businessman surnamed Chin. He visited Guangdong Province on business and developed symptoms

of SARS upon return to Taiwan. Subsequent infections of medical and nursing staff and later nosocomial infections brought about panic to the population.

The Virus

A novel coronavirus, the SARS virus has its RNA(+) with envelop. The length of the nucleic acid is 29,727bp, and the G/C content is about 41%. The genetic structure of [5'-replicase (rep) –spike (S) –envelop (E) –membrane (M)-nucleocapsid (N)-3'] is similar to that of typical coronaviruses. The genome sequences in the rep area are, however, significantly different from those of the coronaviruses that have been found previously. The similarity of genome sequences in other areas was also low[(2)]. Laboratory methods currently used for the diagnosis of SARS include RT-PCR, Real-time RT-PCR, ELISA, IFA and NT. Now that SARS is classified by the Department of Health as Category IV communicable disease[(3)], by regulations of biosafety on laboratory quarantine facilities, SARS specimens must be handled in P2 and above laboratories, and its isolation must be conducted in P3 and above laboratories. The RT-PCR, Real-time PCR and ELISA handling of SARS specimens, therefore, must be handled in P2 and above laboratories. The laboratories of the Center has maintained a close collaboration with the US CDC since the outbreak to establish, based on the information supplied by the US CDC, a real-time RT-PCR diagnosis system for pathogenic agents for the laboratory analysis of the early cases of SARS.

Materials and Methods

Kinds of Specimens and their Handling

As SARS virus causes infection of the respiratory tracts, pathogenic agents are more likely to be detected in pulmonary extracts, sputum and throat swab of

patients at their early stage of infection. Pathogenic agents can also be detected in blood, urine, and fecal specimens of patients at their early and later stages[1,4].

（1）Blood specimens: Sera or plasmas added sodium citrate or EDTA could be used. Specimens were transported at 4℃. They were centrifuged at 2,000 rpm for 10 minutes. The serum so isolated was used.

（2）Throat swabs: Throat swabs were stirred against plastic tubes and squeezed. The fluid was centrifuged under 4℃, at 2,100 xg for 15 minutes. The upper fluid was collected, labeled, dated, and kept at -70℃.

（3）Fecal specimens: One gram of feces was placed in a 15 ml centrifugal tube, added a glass ball and 10 ml of PBS to make a 10% suspension; centrifuged under 4℃, at 2,100 xg for 15 minutes. The upper fluid was collected and placed in 2-3 cryotubes, labeled, dated, and kept at -70℃.

（4）Sputum: Sputum was mixed with 0.9% NaCl (containing 1% N-Acetylcysteine) at a ratio of 1:2; stirred and placed for 30 minutes; centrifuged under 4℃, at 2,100 xg for 15 minutes. The upper fluid was collected.

Probes and Primers

The probes and primers supplied by the US CDC, CDC-1F/ CDC-1R/ CDC1-Probe, and CDC-2F/ CDC-2R/ CDC2-Probe (CDC catalogue #KT0051, were used. The CDC-1 primer was for the augmentation of SARS viruses at the Pol zone; and CDC-2 primer was for the augmentation of SARS viruses at the N zone[5]. The primers of the German-made SARS test reagent kits were for the augmentation of SARS viruses at the NS (non-structure) zone.

Establishment of SARS Virus Standards

The SARS virus isolated by the Laboratory was used as a template. The CDC-1F/CDC-1R, CDC-2F/CDC2-R were used as primers to augment by RT-PCR the genome sections of 81 bps and 78 bps. The genome sections were connected to pGEM-T (pGEM-T Easy Vector, Promega) by molecular biological genetic engineering methods with T4 ligase. The already connected sections were placed into JM109 *E. coli*, and, by using the Ampicillin-resistance genes of the sections, screened with Ampicillin. The screened colonies were augmented in large quantity at 37℃, collected the DNA's, and tested their O.D. 260 value, and diluted to 0.2~2 x106 copies/ul.

Extraction of RNA

RNA was purified with QIAmp Viral RNA kit of the QIAGEN. 140 ul of the specimen was taken and added 560 ul of buffer AVL, and placed under room temperature for 10 minutes. It was added again 560 ul of absolute alcohol for vortexing. The mixed solution was washed twice with buffer AW in QIAmp spin column to dissolve RNA with 80℃ pure water (RNase free). The virus RNA so prepared could be used for reverse transcription polymerase chain reaction.

Real-Time RT-PCR Analysis

ABI 7000 was used for analysis. 5 ul of RNA was added to TaqMan one-step RT-PCR Master Mix Reagents (SN: 4309169). The primer concentration was 400 nM, the probe concentration was 200 nM. TaqMan exogenous internal control (SN: 4308323) was added for reaction at 48℃ for 30 minutes. AmpliTaq DNA was activated at 95℃ for 10 minutes. PCR reaction was conducted for 40 cycles: denature at 95℃ for 15 seconds, and annealing-extension at 60℃ for one minute. Fluorescent signals were

collected in the process of annealing-extension. ABI Prism SDS soft was used for analysis.

The German-made ARTUS test reagent was used together with the Roch LightCycler for analysis. 5 ul of RNA was added to Coronavirus LC Master mix of Mg2+ of 3.75 mM concentration and Coronavirus internal control (IC) of 0.5 ul/reaction. The total volume at the end was 20 ul. RT reaction was conducted at 50℃ for 30 minutes; polymerase was activated at 95℃ for 10 minutes; and PCR reaction was conducted for 50 cycles: denature at 95℃ for two seconds, annealing at 55℃ for 12 seconds, and extension at 72℃ for 10 seconds. Fluorescent signals were collected in the process of annealing, and cooling was done at 40℃ for 30 seconds.

RT-PCR Analysis

The one-step RT-PCR Kit of QIAGEN (Cat No 210212) was used. 5X QIAGEN one-step RT-PCR buffer, dNTP Mix (10 mM), 5X Q-solution, and enzyme Mix were added. Primers F2/R1 and 5 ul of viral RNA (F2: 5'-CTAACATG CTTAGGA TAATGG-3', R1: 5'-CAGGTA AGCGTAA AATCCATC-3') were finally added[6]. RT was conducted at 50℃ for 30 minutes, polymerase DNA activation at 95℃ for 15 minutes, and PCR for 40 cycles: denature at 95℃ for 30 seconds, annealing at 55℃ for 45 seconds, and extension at 72℃ for one minute[7].

Results

Establishment of a Standard Curve

Establishment of a standard curve is most important in the real-time RT-PCR analysis system. The standard curve of the present study used the molecular cloning method. The CDC-1F/CDC-1R and CDC-2F/CDC-2R were added,

through the RT-PCR augmented sections, to pGEMT-EASY vector by T4 ligase, and then transferred to *E. coli* for keeping and mass multiplication. Carriers of nucleic acid concentration 0.2~2x106 copies/ul were extracted from *E. coli*. In each reaction, 5 ul was added to make the standard curve stay around 1~107 copies. CDC1 and CDC2, for their sensitivity to probes and PCR efficiency, could detect one copy of nucleic acid. When analyzing with SDS soft, the Delta Rn threshold was set at 0.20. Fluorescent value higher than this threshold was considered positive, and negative when the value was lower. When the Delta Rn threshold was set at 0.20 in the standard curve, the different numbers of the fluorescent signals produced under 1~106 copies concentrations were defined as Ct (Cycle threshold) (Figures 1 and 2). The fluorescent values produced by higher concentration carriers under fewer PCR cycle numbers were higher than the threshold; and lower concentrations produced the opposite result. When drawing linear chart with Ct value and Log copies, it was suggested that the slope value should be close to –3.3, Y-Intercept value close to 40, correlation value (R2) close to 1.0 (Figures 1 and 2). In assessing CDC1 and CDC2 in the standard curve of the real-time RT-PCR analysis system, it was noted that under similar reaction conditions and environment, the fluorescent values detected by CDC2 in the real-time RT-PCR system were stronger and of higher sensitivity. The standard curve of the German-made Artus reagent was around 10~104 copies. By two differentials, the fluorescent values were the most optimal, and the R-value was 1 (Figure 3).

Sensitivity Analysis

The SARS virus strains of already known concentration supplied by the US CDC were used for sensitivity assessment. The SARS virus RNA was extracted with the QIAGEN ViralAmp test reagent kit. It was diluted to a

concentration of 0.1~106 copies, and analyzed with CDC1, CDC2, ARTUS and conventional RT-PCR. It was found that, with CDC1, CDC2 and ARTUS, the sensitivity could be as high as 1 RNA (Table 1). By using Qiagen one-step RT-PCR for RT-PCR augmentation and analyzed with agar electrophoresis, the sensitivity could be as high as 10 copies RNA (Figure 4), 100~1,000 times higher than the sensitivity of the conventional RT-PCR. Conventional RT-PCR could only detect 1,000~10,000 copies RNA.

Clinical Analysis of Specimens

Specimens of suspected SARS cases sent by various hospitals were analyzed concurrently with real-time RT-PCR, CDC1, CDC2, ARTUS, QIAGEN one-step RT-PCR and agar electrophoresis. In the 15 throat swabs, findings of the real-time RT-PCR were relatively consistent (Table 2). With the exception of specimen 814, which was found negative by all methods, was found positive by the CDC-2 primer and probe. Specimens 768 and 808 were found negative by RT-PCR augmentation and agar electrophoresis with the QIAGEN test reagent kit, was found positive by the real-time RT-PCR system. As the sensitivity of the QIAGEN test reagent kit was 10 copies RNA, when the amount of viruses was low, there could be false negative.

Discussion

The real-time RT-PCR system was most useful in the present SARS outbreak. By using PCR techniques and detection of fluorescent signals, the sensitivity of antibody detection was highly augmented. SARS viruses are hard to detect in the early stage of infection. Its symptoms are similar to those of common cold. Studies have shown that SARS viruses develop communicability until after fever. Therefore, in addition to x-ray diagnosis, fever and dry coughing are

some index symptoms. However, many communicable diseases also show symptoms of fever and coughing, SARS is, therefore, panicky to many.

As it is, in the early stage of infection, viruses can be detected in the pulmonary wash, throat swab and sputum of patients, the Laboratory used from the beginning IN2/IN4 and F2/R1 primers and NEST RT-PCR method for laboratory testing. This method, however, should be repeated twice, chances of contamination were higher. Its sensitivity was lower because it had to be analyzed by agar electrophoresis. The entire process was time-consuming. For developing a more rapid testing method, the Laboratory initially used the ABI primer express soft to design a set of probe and primer. It was found through testing that the maximum sensitivity of the set was only 10 copies RNA. Later, the US CDC helped the Laboratory develop three sets of probes and primers, CDC1, CDC2 and CDC3. By sensitivity and specificity tests, it was found that CDC1 and CDC2 were better. Their sensitivity was as high as 1 copy RNA.

By regulations of the World Health Organization, definition of SARS positive is, 1) positive in at least two different clinical specimens or one clinical specimen collected on two or more different days; and 2) positive of the original specimen by two different tests or repeated PCR test. In the process of PCR operation, each batch of specimens should include adequate number of negative and positive controls to produce expected results. In extracting nucleic acid, positive and negative controls should be added at the same time; and in PCR operation, one water-like control should be added. In the present study, reading of positive reactions followed entirely the standards of the WHO. A clinical specimen was decided positive when it was found positive by CDC1, CDC2, ARTUS, real-time RT-PCR, and conventional RT-PCR. Specimens

collected from different parts of a patient were decided positive when they were found positive by real-time RT-PCR or RT-PCR. To be cautious, PCR positive cases were reviewed by their symptoms and history of contact.

By the results of the study, the sensitivity of CDC2 was found to be higher than that of CDC1 and ARTUS. In the testing of clinical specimens, it could be noted, for instance in specimen 814, by the real-time RT-PCR system using CDC2, the result was weak positive; whereas by CDC1 and ARTUS, the result was negative. In the analysis of SARS of the Laboratory, it occurred sometimes that though the real-time analysis by using CDC2 was positive, testing by other analysis systems was negative. To avoid false negative, it was suggested to use another testing system for confirmation.

When conducting conventional RT-PCR and agar electrophoresis, it was noted that QIAGEN one-step RT-PCR test reagent kit could directly enlarge augments by F2/R1 to 40 cycle numbers, and the sensitivity was equivalent to nest RT-PCR, and could detect 10 copies RNA. When SARS viruses were used for sequence dilution to compare the conventional RT-PCR and QIAGEN one-step RT-PCR test reagent kit, it was found that the conventional RT-PCR could detect 10,000 copies, and the sensitivity was lower than that of QIAGEN one-step RT-PCR (data not shown). Nest PCR could improve sensitivity to 1,000 times to 10 copies RNA. In the analysis of real-time RT-PCR findings, the Delta Rn threshold should be fixed to avoid errors in each experiment. In the present study, the Delta Rn threshold was set at 0.20, the R2 value in the standard curve was larger than 0.99, and the slope value was close to –3.3, to indirectly set the RNA copies of SARS viruses in specimens.

Sensitivity of SARS testing depends on the kinds of specimens and the time the specimens are collected, a true SARS case could show negative in PCR testing. Accuracy of testing could be improved if more specimens were

collected or specimens are collected from different parts of the body. The probes and primers used for RT-PCR and real-time RT-PCR testing of SARS are designed through software and confirmed of their specificity by genome sequence matching, technical errors or environment such as pollution of laboratory and working tables, could produce false positive. Each positive PCR testing, therefore, should be reconfirmed. The Laboratory, in the RT-PCR and real-time RT-PCR testing, always abides by the three-room principle. Preparation of test reagent for PCR, addition of specimens, and PCR analysis are done in three separate rooms and on different operational tables to avoid any false positive due to the pollution of operational tables.

To assure the accuracy and quality of test result, in the analysis of real-time RT-PCR, negative and positive controls will be added. In addition, internal controls (TaqMan exogenous internal control) are also added to assure the efficiency and accuracy of each reaction. For instance, in the present study of CDC-1, CDC-2 real-time RT-PCR analysis, TaqMan exogenous internal control was added as internal control. In the ARTUS real-time RT-PCR analysis, HPA-Coronavirus LC IC was added as internal control.

The use of real-time RT-PCR techniques in the detection of pathogenic agents in clinical specimens has become a trend. With its rapid, high sensitivity and high specificity, pathogenic agents can be detected from specimens in the early stage of infection to help the control of infection. Many local and international experts are in basic research of SARS virus and development of rapid diagnostic reagents. New drugs and vaccines for SARS are expected soon.

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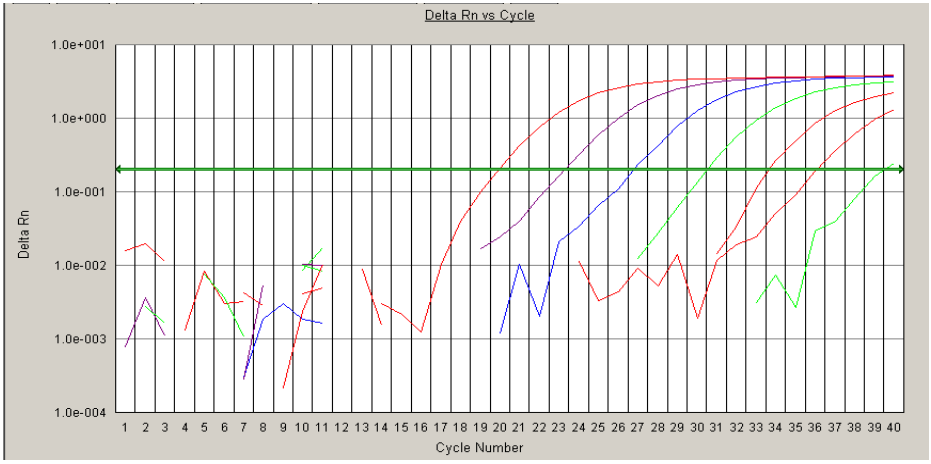
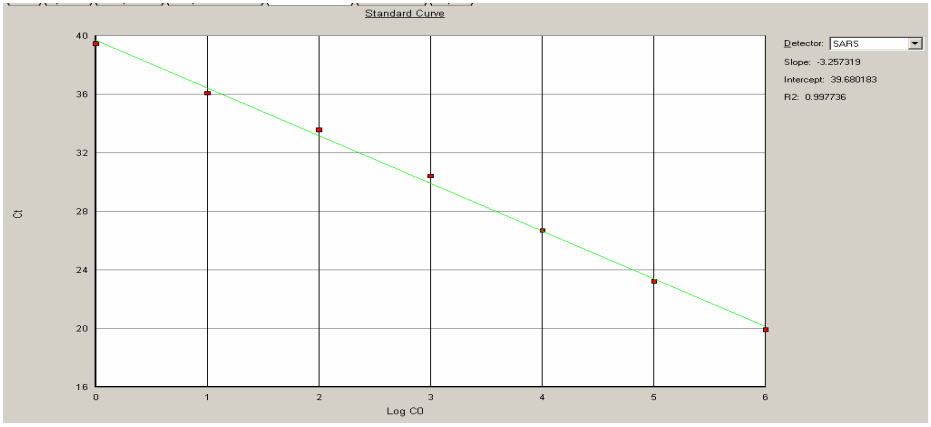
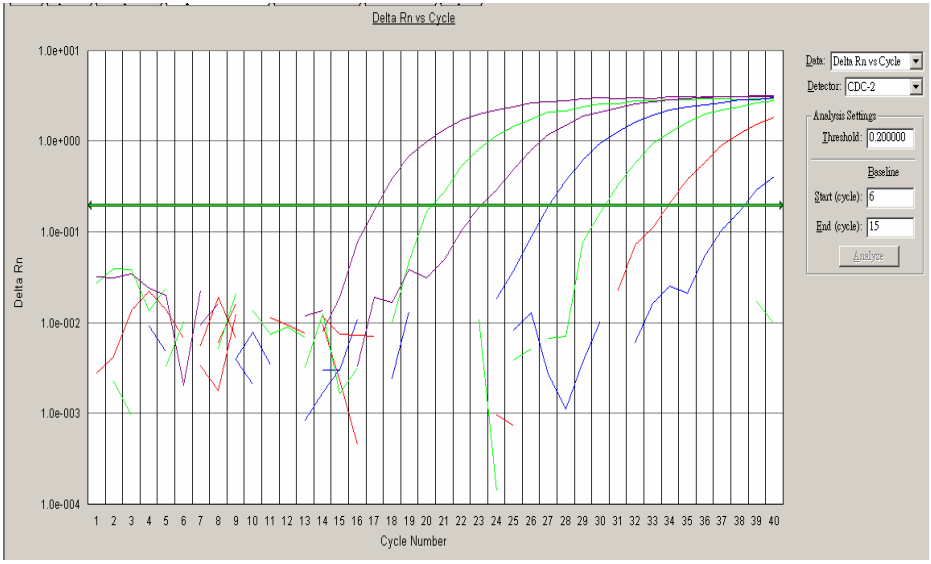
Figure 1. Establishment of Standard Curve for CDC-1 Real-Time System

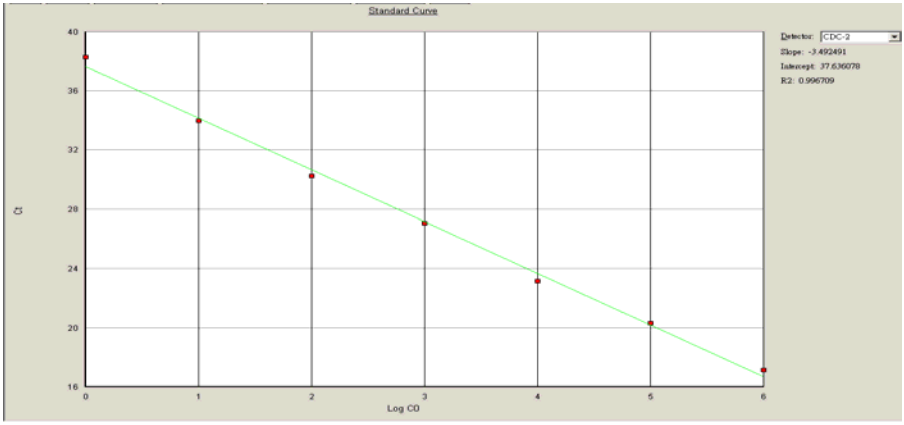




The upper figure shows the standard curve at concentration 106~1 copies, fluorescent signals of Delta Rn, and PCR cycle numbers. The lower figure shows analysis of Ct and Log Co (concentration), in which R2 value is 0.9977, and slope is –3.257.

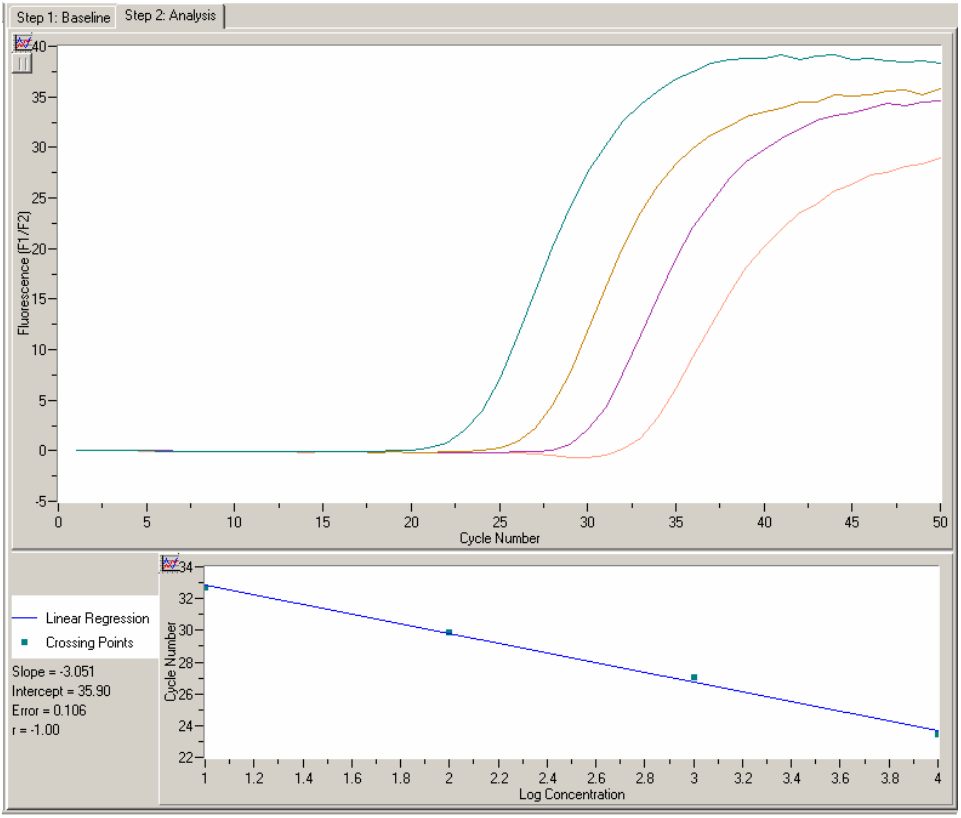
Figure 2. Establishment of Standard Curve for CDC-2 Real-Time System





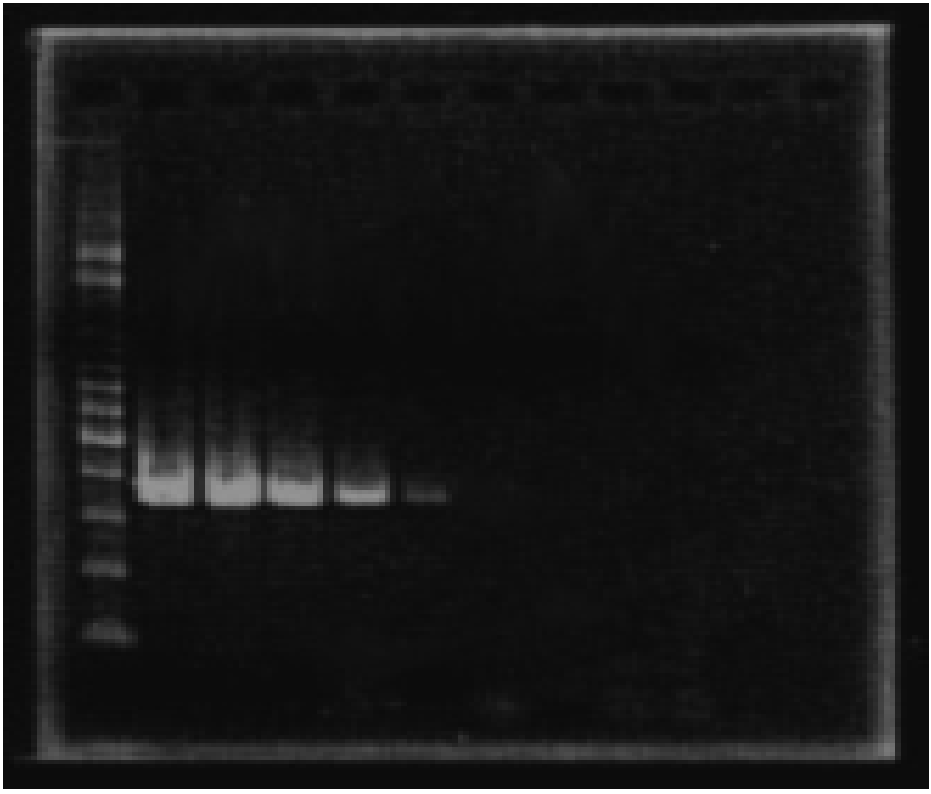
The upper figure shows the standard curve at concentration 106~1 copies, fluorescent signals of Delta Rn, and PCR cycle number. The lower figure shows analysis of Ct and Log Co (concentration), in which R2 value is 0.9967, and slope is –3.4.

Figure 3. Establishment of Standard Curve for ARTUS Test Reagent Kit



The upper figure shows fluorescent signals and PCR cycle numbers. Concentration of the standard curve is 104～10 copies. The lower figure shows cycle number and Log concentration in which, r value is –1.00, and slope is –3.051.

Figure 4. Sensitivity Analysis of RT-PCR Augmentation and Agar Electrophoresis of SARS Viruses



RT-PCR analysis by QIAGEN one-step RT-PCR test reagent kit using F2/R1 as primer. Length of augmented section is 368 bp; of which, the concentration of lane 1-6 is 105～1 copies RNA.

Table 1. Sensitivity of Real-Time RT-PCR and Conventional RT-PCR

Copies number/ul	CDC-1	CDC-2	ARTUS	RT-PCR
1.00E+06	8.9E+05	1.1E+06	2.7E+06	+
1.00E+05	1.5E+05	1.89E+05	4.01E+05	+
1.00E+04	1.45E+04	2.4E+04	1.79E+04	+
1.00E+03	2.2E+03	3.2E+03	1.01E+03	+
1.00E+02	1.72E+02	1.44E+02	9.56E+01	+
1.00E+01	1.3E+01	1.9E+01	2.2E+01	+
1.00E+00	8E-01	1.1E+00	9.5E-01	-
1.00E-01	-	-	-	-

(+ is positive, and – is either negative or undetectable reactions)

Table 2. Analysis of Clinical Specimens

Sample No.	620	648	660	678	738	740	768	788
ARTUS kit analysis	-	+	-	-	-	-	+	+
CDC1	-	+	-	-	-	-	+	+
CDC2	-	+	-	-	-	-	+	+
RT-PCR	-	+	-	-	-	-	-	+
Sample No.	806	808	814	818	928	984	1040	NC
ARTUS kit analysis	+	+	-	+	-	+	+	-
CDC1	+	+	-	+	-	+	+	-
CDC2	+	+	+	+	-	+	+	-
RT-PCR	+	-	-	+	-	+	+	-

15 throat swabs were collected from suspected SARS cases. One negative control was also used. Nucleic acid was extracted for RT-PCR reaction with ARTUS, CDC1, CDC2, real-time system, QIAGEN one-step RT-PCR kits. + is positive, and – is either negative or undetectable reactions.