DEVELOPMENT AND COMPARISON OF THE REAL-TIME AMPLIFICATION BASED METHODS - NASBA-BEACON, RT-PCR TAQMAN AND RT-PCR HYBRIDIZATION PROBE ASSAYS – FOR THE QUALITATIVE DETECTION OF SARS CORONAVIRUS

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Abstract. The aim of this study was to develop a rapid, sensitive and robust procedure for the qualitative detection of SARS coronavirus RNA. Three unique detection formats were developed for real-time RNA amplification assays: a post amplification detection step with a virus-specific internal capture probe based on Taqman (RT-PCR TaqMan assay), hybridization probe (RT-PCR hybridization probe assay) and a real-time assay with virus-specific molecular beacon probes (NASBA-Beacon assay). The analytical sensitivity or reproducibility of the test results among those three assays was compared. All assays yielded results by detecting SARS coronavirus targeting the BNI-1 region in less than 2 hours. RNA detection by all the formats was unaffected by the presence of human sputum. The limits of detection were at least 10 copies of input RNA for both RT-PCR formats (RT-PCR TaqMan and RT-PCR hybridization probe assays), while the NASBA-Beacon assay could detect as little as 1 copy per reaction, with high reproducibility of the coefficient of variation (CV) of <10. These results demonstrate that real-time NASBA provides a rapid and sensitive alternative to RT-PCR for the routine qualitative assay of sputum for SARS corona viral RNA detection.

INTRODUCTION

Severe acute respiratory syndrome (SARS) has recently emerged as a new human disease, resulting in 813 deaths from 8,437 probable cases globally (as of 11 July 2003) (WHO, 2003). The discovery that the virus can be readily isolated in a monkey-kidney cell line was the key to the rapid molecular characterization of this novel coronavirus and the development of diagnostic tests for SARS (Ksiazek *et al*, 2003). The nucleotide sequence of the SARS-associated coronavirus genome differs substantially from sequences of all known coronaviruses (Marra *et al*, 2003; Rota *et al*, 2003).

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SARS-associated coronavirus was proved as the cause of SARS. Inoculation of monkeys with SARS-associated coronavirus from cell cultures caused lower respiratory tract disease, fulfilling Koch's postulate (Fouchier *et al*, 2003). The establishment of a rapid, noninvasive test for this virus is of high priority for monitoring and controlling this disease. Serologic testing for the SARS coronavirus antibody consists of indirect fluorescent antibody testing and enzyme-linked immunobsorbent assays, which have been recently established (Ksiazek *et al*, 2003). Definitive interpretation of negative coronavirus antibody tests is possible only for specimens obtained > 21 days after the onset of fever (CDC, 2003). A reverse transcriptase-polymerase chain reaction (RT-PCR) test specific for RNA from the novel coronavirus can become positive within 10 days of the onset of fever (Drosten *et al*, 2003). The duration of detectable viremia or viral shedding is unknown, and RT-PCR tests on samples collected during convalescence may be negative

(Poon *et al*, 2003).

The key to a rapid diagnosis of SARS coronavirus is sensitivity. Therefore, we have developed a real-time amplification based on three platforms, for the rapid diagnosis of SARS coronaviruses with a sensitivity comparison between each assay. These platforms are, namely, NASBA-Beacon on NucliSens® EasyQ Analyzer (Organon Teknika BV, Boxtel, The Netherlands) (Weusten *et al*, 2002), RT-PCR TaqMan on ABI Prism™ 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) (Monpoeho *et al*, 2000), and RT-PCR hybridization probe assays on LightCycler® (Roche Molecular Biochemicals, Penzberg, Germany) (Jackwood and Sommer, 2002). These probe and primer sets should be compatible with most, if not all, types of real-time amplification machines available in Thailand.

MATERIALS AND METHODS

RNA extraction

Sputum samples from 20 suspected cases were shaken for 30 minutes with an equal volume of acetylcysteinen (10 g/l) and 0.9% sodium chloride. The fluid was then extracted with a viral RNA kit (QIAamp, Qiagen, Hilden, Germany; elution volume, 60μl). For analytical sensitivity evaluation of the three real-time systems, the serial dilution of the amount of known RNA copies transcribed *in vitro* from the BNI-1 fragment was employed to spike into the sputum obtained from a normal person prior to extraction. Dr Christian Drosten of the Bernhard Nocht Institute, Germany, kindly provided the BNI-1 fragment.

Primers and probes

The primers and probes for SARS were designed, based on the known cDNA BNI-1 fragment (Drosten *et al*, 2003). The NASBA-Beacon format was designed using the Primer Premier® 5 software package (PREMIER Biosoft International, Palo Alto, CA), and predicted as the most likely secondary structure for an entered DNA sequence by using the mfold. This mfold was a DNA folding program package (http://www.bioinfo.rpi.edu/~zukerm/), which was designed and maintained by Prof Michael Zuker. In addition, this procedure followed the general rules outlined by the manufacturer. Both the RT-PCR TaqMan and RT-PCR Hybridization probe assay were directly designed using their software package; LightCycler Probe Design® software version 1.0 (Roche Applied Science, ID, USA) and Primer Express® software version 2.0 (Applied Biosystems, CA, USA), respectively. As a final step for all designs, all primer and probe sequences were submitted and screened for specificity using the BLAST® program (http://www.ncbi.nlm.nih.gov/BLAST/). Table 1 illustrates the primer and probe sequences, including protocols for the real-time amplification assay. The NASBA-Beacon sets were synthesized by Eurogentec (Eurogentec S.A.,Seraing, Belgium), and Proligo (Proligo LLC, CO, USA).

NASBA-Beacon assay

Five μl of normal sputum containing the SARS positive control RNA was added to 10 μl of the Nuclisens® Basic kit buffer (bioMérieux, BV, Boxtel, The Netherlands) containing dNTP mix , Tris/HCl, 45% DMSO, $MgCl_2$ 80 mM, 0.4 μM of each primer (ASAR-P1 and ASAR-P2, Table 1) and 0.2 μM of probe (ASAR-MB, Table1). The mixture was incubated at 65ºC for 2 minutes and 41ºC for 2 minutes. Five μl of the enzyme mixture (AMV-RT, RNaseH, and T7 RNA Polymerase) was added, then incubated at 41ºC for 90 minutes in a NucliSens® EasyQ Analyzer (Organon Teknika BV, Boxtel, The Netherlands), and the amplification reaction was monitored real-time. The increase in fluorescent signal was registered during the isothermal amplification.

RT-PCR TaqMan assay

A 5 μl volume of normal sputum containing the SARS positive control RNA was amplified by RT-PCR in a 25 μl reaction, containing TaqMan® 2X Universal PCR Master Mix No AmpErase®UNG, 40 X MutiScribe™ and RNase Inhibitor Mix (Applied Biosystems, CA, USA), 0.4 μM of each primer (ASarF and ASarA2, Table 1) and 0.2 μM of probe (ASarTM, Table 1). The amplification conditions consisted of a reverse transcript step of 48ºC for 30 minutes, followed by an incubation step of 95ºC for 10 minutes. Then, 40 cycles at 95ºC for 15 seconds and 58ºC for 60 seconds, were performed using an ABI Prism™ 7000 sequence detection system (Ap-

Table 1
Primer and probe sequences with protocols.

Protocol	Oligonucleotides	Fragment length	Reagent formulation	Thermal cycling profile
1. NASBA-Beacon format	**ASAR-P2** gATgggTTTCAAAATgAATT ACCA **ASAR-P1** ***AATTCTAATACgACTCACTA*** ***TAggg*** AgAggTAggTTAgTACCCAC AgCA **ASAR-MB** **6FAM-** **cgcgat**gTTCgTgCgTggAT TggCTT**atcgcg** **-Dabcyl**	150 bp	10 µl of reaction buffer Reagent sphere KCl 80 mM 0.4 µM of each primer 0.2 µM of probe 5 µl Enzyme mix 5 µl RNA 20 µl Total volume	65ºC, 2 minutes 41ºC, 2 minutes 41ºC, 90 minutes Fluorescence auto measured at 41ºC
2. RT-PCR TaqMan assay	**ASarS** CACCCgCgAAgAAgCTATTC **ASarA2** CTgTAgAAAATCCTAgCTgg AgAggTA **ASarTM** **FAM-** CgTTCgTgCgTggATTggCT TTg**-TAMRA**	119bp	20 µl of reaction buffer 12.5µl TaqMan® 2X Universal PCR Master Mix No AmpErase®UNG 0.6 µl 40 X MutiScribe™ and RNase Inhibitor Mix Enzyme 0.4 µM of each primer 0.4 µM of probe 5 µl RNA 25 µl Total volume	48ºC, 30 minutes 95ºC, 10 minutes 40 cycles of 95ºC, 15 seconds 58ºC, 60 seconds Fluorescence measured at 58ºC
3. RT-PCR hybridization probe assay	**ASarF** TACCAAgTCAATggTTACCC **ASarR** AgCTggAgAggTAggTT **ASar3FL** ATTCgTCACgTTCgTgCgTg gA **X** **ASar5LC** **LCred640-** ggCTTTgATgTAgAgggCTg TCATgCA**-p**	137 bp	15 µl of reaction buffer 1X LightCycler®-RNA Master Hybridization Probes $Mn(OAc)_2$ 3.25 mM 0.5 µM of each primer 0.4 µM of each probe 5 µl RNA 20 µlTotal volume	61ºC, 20 minutes 95ºC, 2 minutes 65 cycles of 95ºC, 5 seconds 50ºC, 15 seconds 72ºC, 8 seconds Fluorescence measured at 50ºC

Note: Italics letters represent the T7 promoter sequence, and underlined lowercase letters represent the sequence in the stem structure of the Molecular Beacon Probe.

plied Biosystems, CA, USA). The increase in fluorescent signal was registered during the combined annealing and extension steps of the reaction.

RT-PCR hybridization probe assay

The 5 µl of SAR positive control RNA in normal sputum was amplified by RT-PCR in 20 µl of 1X LightCycler®-RNA Master Hybridization Probes (Cat.3018954, Roche Molecular Biochemicals, Mannheim, Germany), containing Tth DNA polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP), Mn (OAc)2 3.25 mM, 0.5 µM of each primer (ASarS and ASarR, Table 1) and 0.4 µM of each probe (ASar3FL and ASar5LC, Table 1). The amplification conditions

Table 2
Comparison of real-time RT-PCR hybridization probe assay, TaqMan assay and NASBA-Beacon assay[a].

BNI-1 fragment (189 bp) of SARS coronavirus		RT-PCR hybridization probe assay			RT-PCR TaqMan assay			NASBA-Beacon assay		
		Cr	CV(%)	Int	C_T	CV(%)	Int	Signal	CV(%)	Int
Triplicate assays of	10^5	21.46	2.91	POS	21.55	3.53	POS	12.72	7.11	POS
	10^5	22.64		POS	20.50		POS	14.67		POS
	10^5	21.63		POS	20.14		POS	13.75		POS
Triplicate assays of	10^4	24.65	2.48	POS	28.08	8.48	POS	13.06	6.20	POS
	10^4	25.62		POS	24.06		POS	11.68		POS
	10^4	25.62		POS	24.65		POS	13.00		POS
Triplicate assays of	10^3	30.73	2.94	POS	31.25	27.43	POS	13.82	5.17	POS
	10^3	30.12		POS	28.42		POS	12.56		POS
	10^3	31.91		POS	17.81		POS	13.68		POS
Triplicate assays of	10^2	35.29	2.74	POS	35.50	13.83	POS	14.30	8.87	POS
	10^2	35.24		POS	29.93		POS	12.46		POS
	10^2	33.62		POS	27.12		POS	12.18		POS
Triplicate assays of	10^1	32.74	3.02	POS	38.71	15.69	POS	9.83	10.47	POS
	10^1	33.78		POS	33.69		POS	11.41		POS
	10^1	34.78		POS	28.19		POS	12.1		POS
Triplicate assays of	1	NEG	ND	NEG	27.84	1.24	NEG	10.94	9.06	POS
	1	NEG		NEG	27.27		NEG	9.12		POS
	1	NEG		NEG	27.89		NEG	10.14		POS
Triplicate assays of	NTC	NEG	ND	NEG	15.78	12.30	NEG	1.26	0.79	NEG
	NTC	NEG		NEG	19.77		NEG	1.25		NEG
	NTC	NEG		NEG	19.62		NEG	1.27		NEG

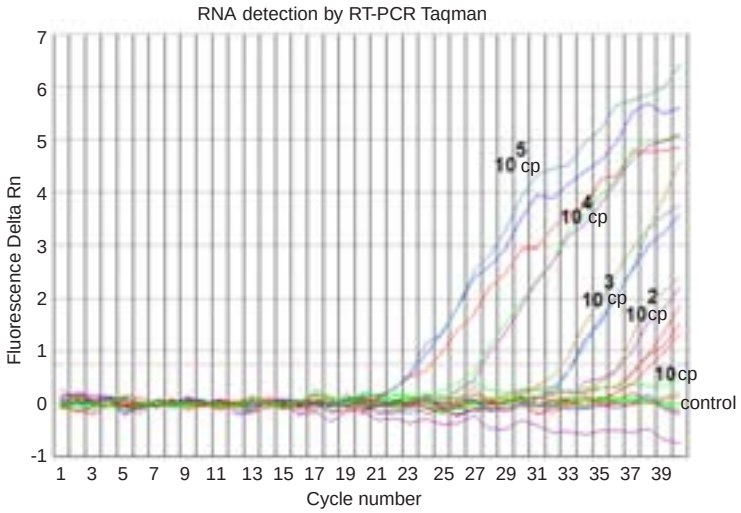
[a]Abbreviation: POS, positive; NEG, negative; Int, interpretation; NTC, sputum with no-template control; C_r, crossing point; C_T, cross talk; CV, coefficient of variation; ND, not determined

consisted of a reverse transcript step of 61ºC for 20 minutes, followed by an incubation step of 95ºC for 2 minutes. Then, 65 cycles of 95ºC for 5 seconds, 50ºC for 15 seconds and 72ºC for 8 seconds were performed using a LightCycler® (Roche Molecular Biochemicals, Penzberg, Germany). The increase in fluorescent signal was registered during the annealing step of the reaction.

RESULTS

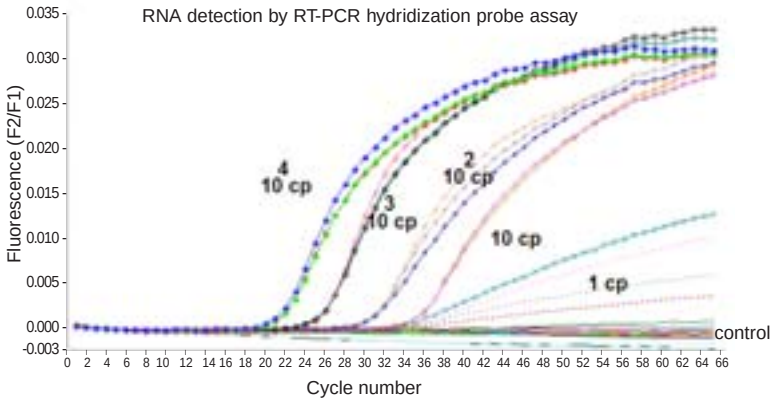
The reproducibility and the analytical sensitivity of the three real-time amplification based methods, namely, NASBA-Beacon, RT-PCR TaqMan and RT-PCR hybridization probe assay for diagnosis of the SARS coronavirus, are compared in Table 2. In addition, RNA detection by any of the three formats, was unaffected by the presence of human sputum (Figs 1-3).

All assays yielded results in less than 2 hours. The limits of detection were at least 10 copies of input RNA for both the RT-PCR formats, RT-PCR TaqMan (Fig 1), and RT-PCR hybridization probe assay (Fig 2); while the NASBA-Beacon assay could detect as little as 1 copy per reaction (Fig 3), with a high reproducibility of the coefficient of variation (CV) of less than 10 (Table 2). Moreover, a positive signal at a low copy number, such as 1 copy, has been shown to be clearly and easily identified on the monitor screen (Fig 3). The signal from the low copy target in the LightCycler and the ABI7000 platform was difficult to distinguish from the negative control (H_2O) (Figs 1 and 2).



cp = copies of input RNA

Fig 1–RNA detection was unaffected by the presence of human sputum. The limits of detection were at least 10 copies of input RNA for RT-PCR TaqMan probe assays.



cp = copies of input RNA

Fig 2–RNA detection was unaffected by the presence of human sputum. The limits of detection were at least 10 copies of input RNA for RT-PCR hybridization probe assay.

DISCUSSION

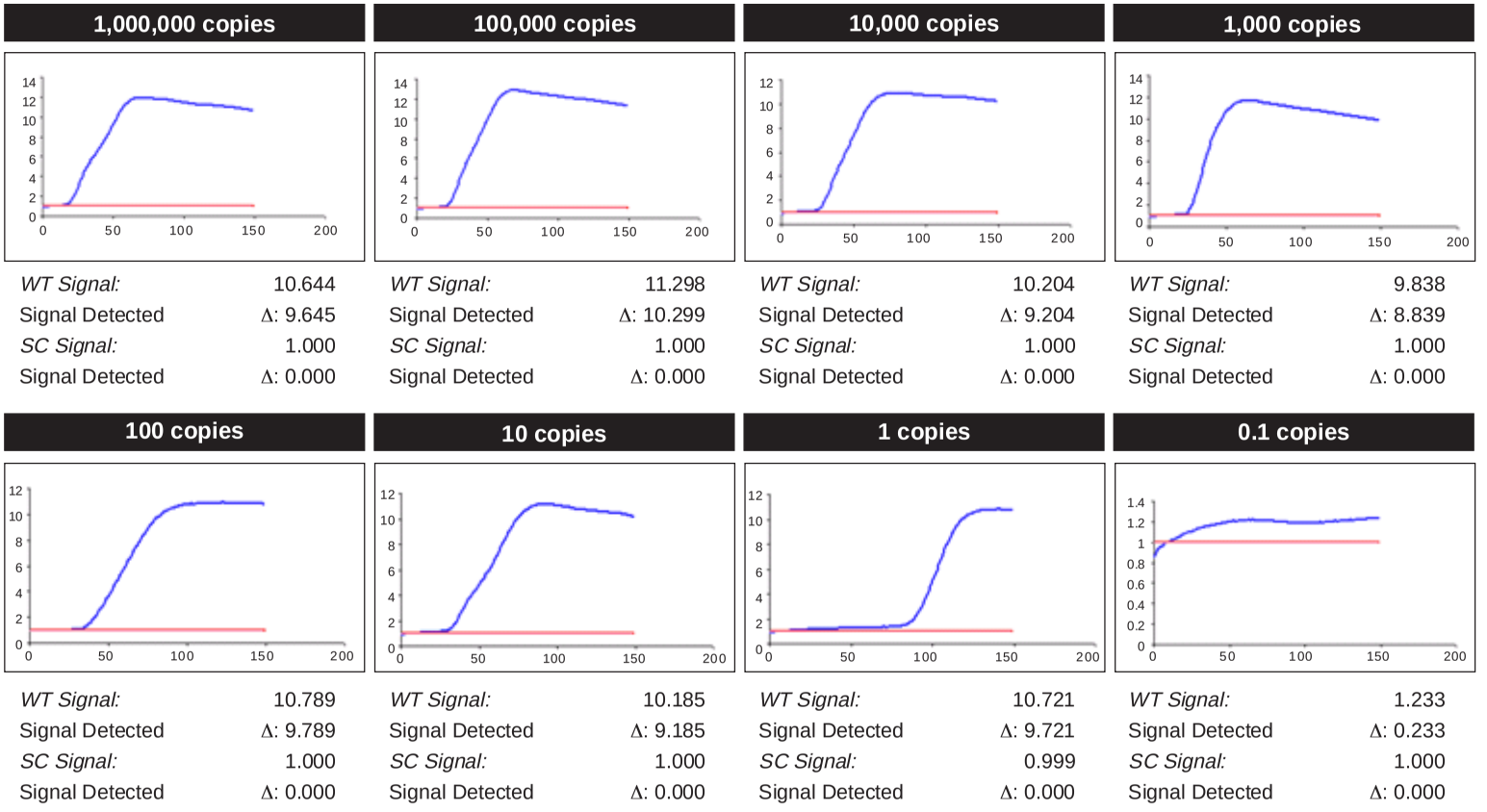
The establishment of a rapid molecular qualitative assay for SARS coronavirus is of high priority for monitoring and controlling this disease, since serological testing for SARS coronavirus antibody is possible only from specimens obtained more than 21 days after the onset of fever (CDC, 2003). Serological testing would then play a key role as a confirmatory test, but not for rapid diagnosis.

These results demonstrate that real-time NASBA provides a rapid and sensitive alternative to RT-PCR for the routine qualitative assay of sputum SARS corona virus RNA detection. The NASBA-Beacon was the least expensive of the instruments. The only drawback to the NASBA-Beacon NucliSens® EasyQ Analyzer so far is the system not equipped with quantification software. We could only perform qualitative assays with the NASBA-Beacon. However, we could easily perform quantitative assays without any difficulty on the real-time RT-PCR using either LightCycler or ABI7000 instruments .

Analytical sensitivity alone does not predict the ability of the assays to detect SARS coronavirus in a clinical sample. Before these assays can be used on a wide-scale for rapid diagnosis, much more has to be done, particularly in evaluating these assays with real clinical samples, which are not available in Thailand. Although these assays were tested with 20 probable SARS cases in Ramathibodi Hospital, none showed a positive result. None of the viruses could be isolated from these samples. Finally, during preparation of this manuscript, a serological test for SARS coronavirus was not available in Thailand, and all 20 cases were discharged from the hospital without complications.

In summary, we have developed three real-time amplification based methods, which could be almost, if not completely, compatible with the real-time instruments available in the laboratories of this country. The overall process from probe primer design to evaluation with controls and specimens was finished in less than a month. Thus, the real-time amplification methods would

RNA detection by NASBA-Beacon Assay



Key:
On all graphs, the abscissa or x-axis represents the number of cycles. The ordinate or y-axis represents the fluorescent signal.
The dense line is the measured signal
The light line is the control signal

Fig 3–The sensitivity of the NASBA-beacon assay could detect as little as 1 copy per reaction. RNA detection was unaffected by the presence of human sputum.

be suitable to serve the need for the development of molecular diagnosis testing on emerging diseases, like SARS Coronavirus. NASBA-Beacon on the NucliSens® EasyQ Analyzer was 10 fold more sensitive than RT-PCR TaqMan on the ABI7000 and RT-PCR hybridization probe assay on the LightCycler.

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REFERENCES

Centers for Disease Control and Prevention. Severe Acute Respiratory Syndrome (SARS) and coronavirus testing–United States, 2003. *JAMA* 2003; 289: 2203-6.

Drosten C, Gunther S, Preiser W, *et al.* Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med* 2003; 348: 1967-76.

Fouchier RA, Kuiken T, Schutten M, *et al.* Etiology: Koch's postulates fulfilled for SARS virus. *Nature* 2003; 423: 240.

Jackwood DJ, Sommer SE. Identification of infectious bursal disease virus quasispecies in commercial vaccines and field isolates of this double-stranded RNA virus. *Virology* 2002; 304: 105-13.

Ksiazek TG, Erdman D, Goldsmith CS, *et al.* A novel coronavirus associated with severe acute respiratory syndrome. *N Engl J Med* 2003; 348: 1953-66.

Marra MA, Jones SJ, Astell CR, *et al.* The Genome sequence of the SARS-associated coronavirus. *Science* 2003; 300: 1399-404.

Monpoeho S, Dehee A, Mignotte B, *et al.* Quantification of enterovirus RNA in sludge samples using single tube real-time RT-PCR. *Biotechniques* 2000; 29: 88-93.

Poon LL, Wong OK, Chan KH, *et al.* Rapid diagnosis of a coronavirus associated with severe acute respiratory syndrome (SARS). *Clin Chem* 2003; 49: 953-5.

Rota PA, Oberste MS, Monroe SS, *et al.* Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* 2003; 300: 1394-9.

Weusten JJ, Carpay WM, Oosterlaken TA, van Zuijlen MC, van de Wiel PA. Principles of quantification of viral loads using nucleic acid sequence-based amplification in combination with homogeneous detection using molecular beacons. *Nucleic Acids Res* 2002; 30: e26.

WHO Update on SARS: Cumulative number of reported probable cases of SARS. Available from: http://www.who.int/csr/sars/country/2003_07_11/en/