SARS – Urbani Coronavirus

Assay no. [279]

TIB MOLBIOL

Reports of the Bernhard-Nocht-Institute in Hamburg and the US Centers for Disease Control and Prevention (CDC) indicate that a previously unrecognized coronavirus – a virus family associated mainly with the common cold – may be the cause of severe acute respiratory syndrome (SARS). Wordwide there were 3169 cases and 144 deaths reported (WHO, 04/14/2003).
New information on this virus and diagnostics may be available from:

Bernhard-Nocht-Institute Hamburg, Germany www.bni-hamburg.de	CDC SARS page www.cdc.gov/ncidod/sars/
Robert-Koch-Institute Berlin, Germany www.rki.de	WHO SARS information www.who.int/csr/sars/en/

_ Coronavirus (common cold) _

Structure
Spherical or pleomorphic enveloped particles containing single-stranded (positive-sense) RNA associated with a nucleoprotein within a capsid comprised of matrix protein. The envelope bears club-shaped glycoprotein projections.

Classification
Coronaviruses (and toroviruses) are classified together on the basis of the crown or halo-like appearance of the envelope glycoproteins, and on characteristic features of chemistry and replication. Most human coronaviruses fall into one of two serotypes: OC43-like and 229E-like.

Multiplication
The virus enters the host cell, and the uncoated genome is transcribed and translated. The mRNAs form a unique "nested set" sharing a common 3' end. New virions form by budding from host cell membranes.

Pathogenesis
Transmission is usually via airborne droplets to the nasal mucosa. Virus replicates locally in cells of the ciliated epithelium, causing cell damage and inflammation.

Normal Clinical Presentation
Coronaviruses cause acute, mild upper respiratory infection (common cold).

_ RNA preparation and RT-PCR protocols _

The following protocol and primer sequences[&] were communicated by Christian Drosten and S. Günther, BNI
Additional information might be available from C. Drosten (drosten@bni-hamburg.de / +49-40-42818-421).

The primers indicated with * are listed at: www.who.int/csr/sars/primers/en/ ; sequences and the diagnostic procedure is published in NEJM, see: http://content.nejm.org/cgi/reprint/NEJMoa030747v2.pdf

_ RNA preparation from sputum _

- ► Add 1 volume of 2X N-acetylcysteine buffer (0.9% NaCl infusion solution containing 10 g/l N-acetylcysteine); shake slowly for 30 min,
- ► spin 600 µl of this solution in a bench top centrifuge at 10,000 g for 3 min
- ► add 140 µl of the supernatant into 560 µl buffer AVL, Qiagen viral RNA kit
  in addition, re-dissolve the cell pellet in another 560 µl buffer AVL and add 140 µl water
- ► proceed according to instructions of the Qiagen viral RNA kit
- ► elute in 60 µl of 80°C preheated buffer AVE (or water)

SARS Coronavirus RT-PCR assays

_RT-PCR-Primers from the WHO network laboratories and from the BNI _

Table 1

[279.79/80]	pan-Corona-PCR: IN-2/IN-4 ≈ 452 bp // IN-6/IN-7 = 440 bp		AF124990	Tm
IN-2$*	ggg TTg ggA CTA TCC TAA gTg TgA	S	331-354	58.7°C
IN-4$*	TAA CAC ACA AAC ACC ATC ATC A	A	784-763	52.2°C
IN-6$*	gg TTg ggA CTA TCC TAA gTg TgA	S	332-354	55.5°C
IN-7$*	CCA TCA TCA gAT AgA ATC ATC ATA	A	771-748	41.5°C
[279.77/80]	**SAR1s/SAR1as: 121 bp, faint additional at 290 bp**		**wSARS_1**	**Tm**
SAR1s&*	CCT CTC TTg TTC TTg CTC gCA	S	15271-291	59.1°C
SAR1as&*	TAT AgT gAg CCg CCA CAC ATg	A	15391-371	57.7°C
[279.78/80]	**BNIoutS2/BNIoutAs: 190 bp // BNIinS/BNIinAs: 109 bp**		**wSARS_1**	**Tm**
BNIoutS2&*	ATg AAT TAC CAA gTC AAT ggT TAC	S	18153-176	52.4°C
BNIinS&*	gAA gCT ATT CgT CAC gTT Cg	S	18201-220	54.6°C
BNIinAs&*	CTg TAg AAA ATC CTA gCT ggA g	A	18309-288	51.8°C
BNIoutAs&*	CAT AAC CAg TCg gTA CAg CTA C	A	18342-321	53.4°C
[279.x]	**Government Virus Unit Hong Kong: 286 bp**		**wSARS_1**	**Tm**
COR-1*	CAC CgT TTC TAC Agg TTA gCT AAC gA	S	15318-343	60.8°C
COR-2*	AAA TgT TTA CgC Agg TAA gCg TAA AA	A	15628-603	60.4°C
[279.x]	**Lancet, Peiras et al. : 182 bp**		**wSARS_1**	**Tm**
Peiras_F*	TAC ACA CCT CAg CgT Tg	S	18041-057	49.1°C
Peiras_R*	CAC gAA CgT gAC gAA T	A	18222-207	48.2°C

Primers indicated with $ were recommended by the CDC (16.03.2002), primers& are from C. Drosten, BNI

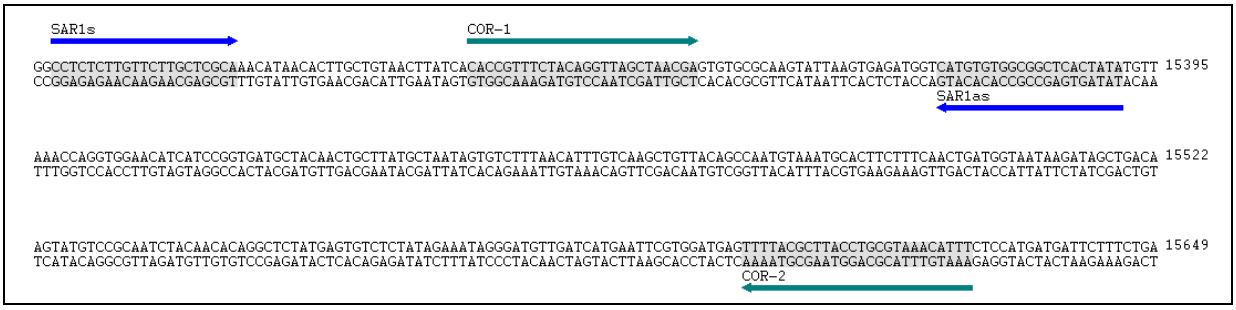


Figure 1

Proposed reaction conditions for Nested RT-PCR (SAR and BNI-primers&)

Table 2

First Round PCR		RT-PCR-Parameters	Second Round PCR		PCR-Parameters
Superscript II/Platinum Kit		45°C, 30 min / 95°C, 3 min	Platinum Taq, 10966-018		95°C, 3 min
Invitrogen 10928-042		**10 cycles:**	**50 µl total volume**		**10 cycles:**
20 ul total volume		95°C 10 sec	10x buffer	5 µl	95°C 10 sec
2x reaction mix	10 µl	60°C 10 sec (drop 1°C)	dNTP mix	4 µl	60°C 10 sec (drop 1°C)
50mM $MgSO_4$	0.5 µl	72°C 20 sec	$MgCl_2$ 50mM	2.5µl	72°C 20 sec
Primers 10 µM	2x 0.5 µl	**40 cycles**	Primers 10 µM	2x 0.5 µl	**25 cycles**
RT/Taq Mixture	0.4 µl	95°C 10 sec	Platinum Taq	0.2 µl	95°C 10 sec
add water ad 18µl:	6.1 µl	56°C 10 sec	add water ad 49µl:	36.3 µl	56°C 10 sec
RNA extract	2 µl	72°C 20 sec	First-Round-PCR	1 µl	72°C 20 sec

Protocol from: www15.bni-hamburg.de/bni/bni2/neu2/getfile.acgi?area_engl=news&pid=620
Analysis: Run 1% agarose gels as cellular background often produces smears, which could be interpreted as PCR-products.

SARS Coronavirus RT-PCR assays

_RT-TaqMan PCR from BNI _

Table 3

[279.81]	TaqMan Assay: 99 bp		ʷSARS_1	Tm
TMSARS1[&*]	TTA TCA CCC gCg AAg AAg CT	S	18187-206	58.3°C
TMSARAs1[&*]	gTA ggT TAg TAC CCA CAg CAT CTC TAg T	A	18285-258	57.7°C
TMSARP1[&]	FAM-TCgTgCgTggATTggCTTTgATgT-TAMRA	S	18218-241	68.1°C

Table 4

[279.82][§]	TaqMan Assay: 78 bp		ʷSARS_1	Tm
TMSARS1[&*]	TTA TCA CCC gCg AAg AAg CT	S	18187-206	58.3°C
TMSARAs2[&*]	CTC TAg TTg CAT gAC AgC CCT C	A	18264-243	57.5°C
TMSARP1[&]	FAM-TCgTgCgTggATTggCTTTgATgT-TAMRA	S	18218-241	68.1°C

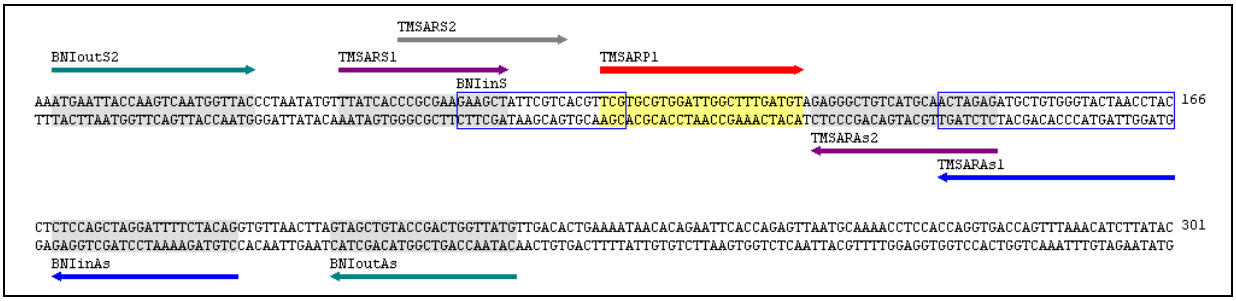


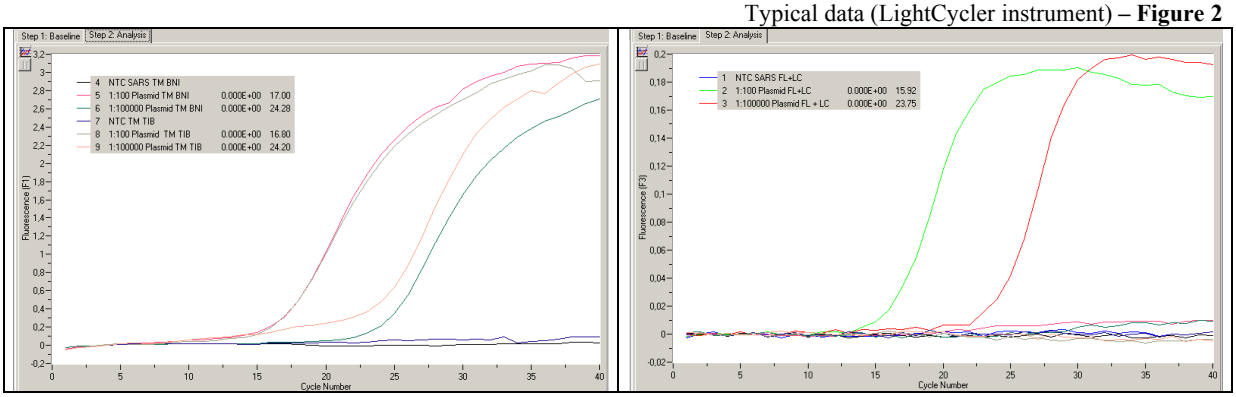
Figure 2

Reaction conditions for the TaqMan-Probe-based assay

Table 5

TaqMan RT-PCR		RT-PCR-Parameters	Instruments
	25 ul total volume	45°C, 15 min	**Protocol tested on:**
Reagents:	2x reaction mix 12.5 µl	95°C, 3 min	
Superscript II/Platinum Kit	BSA, 1mg/ml 1.0 µl		LightCycler (Roche Diagnostics)
Invitrogen 10928-042	50mM $MgSO_4$ 1.2 µl		SDS7000 (ABI)
Non-acetylated BSA	Primers 10 µM 2x 0.5 µl	**40 cycles:**	
	Probe 10 µM 0.6 µl	95°C 10 sec	Protocol supplied by C. Drosten
Primers and probes :	RT/Taq Mixture 0.6 µl	58°C 30 sec	Published in NEJM see [§]
Set: SARS_81 or SARS_82	add water ad 20µl: 3.1 µl		
	RNA extract 5 µl		

Typical data (LightCycler instrument) – **Figure 2**



Left panel: LightCycler data obtained with primers BNIoutS and BNIoutAs and TaqMan probe (channel F1) and plasmid target. **Right panel**: LightCycler data obtained with primers BNIoutS and BNIoutAs and hybridization probes (channel F3).

Disclaimer: This is not a diagnostic 'kit'. Primers and procedures were not validated by TIB MOLBIOL; primers and probes were verified using a cloned BNI-1 fragment. Reagents from other suppliers (Qiagen & Invitrogen) according to BNI protocol. The purchase of these products does not include the rights to practice the Polymerase Chain Reaction ("PCR") and does not convey any right for its use in clinical diagnostic applications.

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SARS Coronavirus RT-PCR assays

RT-LightCycler-PCR (hybridization probes)

Table 6

[279.83]	LightCycler Assay: 190 bp		[w]SARS_1	Tm
BNIoutS2[&*]	ATg AAT TAC CAA gTC AAT ggT TAC	S	18153-176	52.4°C
TMSARS1[&*]	TTA TCA CCC gCg AAg AAg CT	S	18187-206	58.3°C
BNIoutAs[&*]	CAT AAC CAg TCg gTA CAg CTA C	A	18342-321	53.4°C
SARS FL	ACAgCATCTCTAgTTgCATgACAgCCC X	A	18271-245	65.4°C
SARS 705	CTACATCAAAgCCAATCCACgCACgA p	A	18243-218	67.3°C

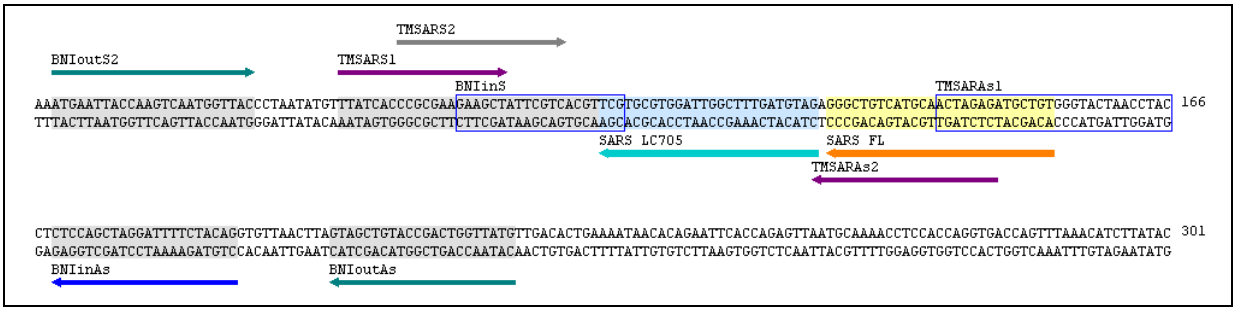

Figure 3

Tested reaction conditions for the LightCycler assay (DNA)

Table 7

PCR		PCR-Parameters	Instruments
Reagents: Roche Diagnostics FastStart Hybridization probes **Primers and probes** : Set: SARS_83	**20 ul total volume** 5x reaction mix 4 µl 25 mM $MgCl_2$ 1.6 µl Primers 10 µM 2x 1 µl Probes 3 µM 2x 1 µl add water ad 20µl: 8.4 µl sample DNA : 2 µl	95°C, 10 min **40 cycles:** 95°C 10 sec 56°C 8 sec 72°C 14 sec **Melting curve:** 95°C 20 sec 40°C 20 sec to 85°C with 0.2°C/sec	**Protocol tested on:** LightCycler (Roche Diagnostics)

_ Target sequence_

Table 8

Amplicon sequence (BNI-1)	TACCGTAGACTCATCTCTATGATGGGTTTCAAAATGAATTACCAAGTCAATGGTTAC CCTAATATGTTTATCACCCGCGAAGAAGCTATTCGTCACGTTCGTGCGTGGATTGGC TTTGATGTAGAGGGCTGTCATGCAACTAGAGATGCTGTGGGTACTAACCTACCTCTC CAGCTAGGATTTTCTACAGGTGTTAACTTAGTAGCTGTACCGACTGGTTATGTTGAC ACTGAAAATAACACAGAATTCACCAGAGTTAATGCAAAACCTCCACCAGGTGACCAG TTTAAACATCTTATACC

Sequence BNI-1 from : www.who.int/csr/sars/primers/en/
[w]Complete preliminary sequence (SARS_1=29727 bp) see: www.cdc.gov/ncidod/sars/pdf/nucleoseq.pdf

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RT-PCR-Primer (predefined sets of primers and probes, each 5 nmol)

The following primer-sets are predefined according to the published sequences. These sets are ready for shipping.

Table 9

SARS_77 6 Primers, 5 nmol	SARS_78 4 Primers, 5 nmol	SARS_79 4 Primers, 5 nmol	SARS_80 10 Primers, 5 nmol	SARS_81 Primers, TaqMan	SARS_82§ Primers, TaqMan	SARS_83 LC 1 nmol
		IN-2	IN-2			
		IN-4	IN-4			
		IN-6	IN-6			
		IN-7	IN-7			
SARS1s			SARS1s			
SARS1as			SARS1as			
BNIoutS2	BNIoutS2		BNIoutS2			BNIoutS2
BNIinS	BNIinS		BNIinS			
BNIinAS	BNIinAS		BNIinAS			
BNIoutAS	BNIoutAS		BNIoutAS			BNIoutAS
				TMSARS1	TMSARS1	TMSARS1
				TMSARAs1	TMSARAs2	
				TMSARP1	TMSARP1	
						SARS_FL
						SARS_LC
€ 59,82	**€ 39,88**	**€ 39,88**	**€ 99,70**	**€ 199,00**	**€ 199,00**	**€ 249,00**
US$ 60.00	**US$ 40.00**	**US$ 40.00**	**US$ 100.00**	**US$ 230,00**	**US$ 230,00**	**US$ 279,00**

Dissolve 5 nmol primer or **TaqMan** (lyophilized) **in 500 µl** water (or TE buffer) to obtain a **10 µM solution** (1.000 reactions). Use each 0.5 µl for a 25 µl reaction Dissolve 1 nmol hybridization probes in 330 µl water to obtain a 3 µM solution. Use 1 µl /20 µl reaction. Store dissolved products at 4°C (daily use) or frozen at -20°C.

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Positive samples / Controls

A positive *in vitro*-transcribed RNA sample is distributed by the BNI. Contact: drosten@bni-hamburg.de
We offer a cloned control sequence (DNA) 10^{10} copies, lyophilized. The sequence of the plasmid is shown in figure 4. The plasmid can be used as target to generate *in vitro*-transcribed +strand RNA (RT-PCR test target).

Product No. 30-9279-01 **Table 10**

Dissolve 10^{10} sample in 100 µl. Dilution row from stock solution 10^8. Use 1 µl sample per 20 µl reaction.						
10^8	10^7	10^6	10^5	10^4	10^3	10^2
10^8	10 µl					
	90 µl water					
	10^7	10 µl				
		90 µl water				
		10^6	10 µl			
			90 µl water			
			10^5	10 µl		
				90 µl water		
				10^4	10 µl	
					90 µl water	
					10^3	10 µl
						90 µl water
						10^2

Plasmid: pCR2.1-TOP>bni< * 4200 bp * Ampicillin Resisistance *

cttccgcttc ctcgctcact gactcgctgc gctcggtcgt tcggctgcgg cgagcggtat
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TAGggcgaat tgggccctct agatgcatgc tcgagcggcc gccagtgtga tggatatctg
cagaattcgc cctTACCGTA GACTCATCTC TATGATGGGT TTCAAAATGA ATTACCAAGT Red : Insertion BNI-1
CAATGGTTAC CCTAATATGT TTATCACCCG CGAAGAAGCT ATTCGTCACG TTCGTGCGTG
GATTGGCTTT GATGTAGAGG GCTGTCATGC AACTAGAGAT GCTGTGGGTA CTAACCTACC
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tgggcgct

Figure 4

SARS Coronavirus RT-PCR assays

TIB MOLBIOL

_ New Publications on SARS Corona Virus_

Identification of Severe Acute Respiratory Syndrome in Canada. Poutanen SM, Low DE, Henry B, Finkelstein S, Rose D, Green K, Tellier R, Draker R, Adachi D, Ayers M, Chan AK, Skowronski DM, Salit I, Simor AE, Slutsky AS, Doyle PW, Krajden M, Petric M, Brunham RC, McGeer AJ. : N Engl J Med 2003 Apr 4
http://content.nejm.org/cgi/reprint/NEJMoa030634v2.pdf

Coronavirus as a possible cause of severe acute respiratory syndrome. J S M Peiris, S T Lai, L L M Poon, Y Guan, L Y C Yam, W Lim, J Nicholls, W K S Yee, W W Yan, M T Cheung, V C C Cheng, K H Chan, D N C Tsang, R W H Yung, T K Ng, K Y Yuen, and members of the SARS study group. The Lancet (2003)
http://image.thelancet.com/extras/03art3477web.pdf

A Novel Coronavirus Associated with Severe Acute Respiratory Syndrome. Ksiazek, T.G., Erdman, D., Goldsmith, C., Zaki, S.R., Peret, T., Emery, S., Tong, S., Urbani, C., Comer, J.A.., Lim, W., Rollin, P.E., Nghiem, K.A., Dowell, S., Ling, A.E., Humphrey, C., Shieh, W.-J., Guarner, J., Paddock, C.D., Rota, P., Fields, B., DeRisi, J., Yang, J.Y., Cox, N., Hughes, J., LeDuc, J.W., Bellini, W.J., Anderson, L.J., for the SARS Working Group
http://content.nejm.org/cgi/reprint/NEJMoa030781v2.pdf

[§]Identification of a Novel Coronavirus in Patients with Severe Acute Respiratory Syndrome. Drosten, C., Günther S., Preiser, W., van der Werf, S., Brodt, H.-J., Becker, S., Rabenau, H, Panning, M., Kolesnikova, L., Fouchier, R.A.M., Berger, A. Burguière, A.M., Cinatl, J., Eickmann, M., Escriou, N., Grywna, K., Kramme, S., Manuguerra, J.-C., Müller, S., Rickerts, V., Stürmer, M., Vieth, S., Klenk, H.-D., Osterhaus, A.D.M.E., Schmitz, H. and Doerr, H.-W.
http://content.nejm.org/cgi/reprint/NEJMoa030747v2.pdf

TIB MOLBIOL statement

There is no commercial incentive for a collaboration between the Bernhard-Nocht Institute and the company TIB MOLBIOL. No employee of the Bernhard-Nocht-Institute receives money or other personal benefits from TIB MOLBIOL.

TIB MOLBIOL provides primers and probes for laboratories worldwide, focusing on Real-Time-PCR applications and the development of new PCR assays. Our production facilities operate 24/7 enabling us to provide the scientific community with our products in a reasonable time. We have a long history of collaboration with the Bernhard-Nocht Institute; for example, the diagnosis of the Yellow Fever infection of a camera man in Berlin in August 1999 was performed with primers synthesized by TIB MOLBIOL, Berlin.

In cases of infectious diseases with the potential of a pandemic spread we try to provide new information and new PCR systems as rapidly as possible. This had been the case after the Anthrax attacks in autumn 2001 and this will also be the case in any future similar circumstances.

TIB MOLBIOL does not manufacture diagnostic kits and we disclose sequence information. We prefer to publish sequences with a diagnostic relevance to enable the scientific community to exchange and discuss PCR results.
Our products should not be used as 'diagnostic kits' - they are not validated for diagnostic procedures and they should be used by skilled personnel only. PCR analysis alone is not sufficient for SARS diagnostics.
Handling of SARS-suspected clinical sampled requires a biosafety laboratory environment.

We deposited aliquots of the relevant SARS primers at all Roche Diagnostics offices in Far East before first SARS primer requests arrived.
The price for the synthesis of the relevant sequences will be according to our normal custom synthesis or even lower (as it is the case for the TaqMan/LC assays). We do not charge 'consultant' fees.

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TIB MOLBIOL, Berlin

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SARS Coronavirus RT-PCR assays

TIB MOLBIOL

BNI statement - (ProMED-mail: SARS - WORLDWIDE (46): DIAGNOSTIC TEST; 13.04.2003)

The **Bernhard-Nocht Institute (BNI)** has developed and evaluated different RT-PCR tests for **SARS**-associated Coronavirus (for simplicity, hereinafter called SARSV, note that this in not an officially proposed name). It has been our policy to make available all our tests for SARSV before publication to rapidly provide reliable diagnostic tools (see ProMed posting SARS - WORLDWIDE (19): ETIOLOGY). Protocols of the BNI PCR's are available from www.bni-hamburg.de or, together with that of other institutions, at the WHO homepage http://www.who.int/csr/sars/primers/en/.

BNI currently recommends two different RT-PCR's for SARSV:
(1) A nested PCR, primers BNIoutS2, BNIoutAs; BNIinS, BNIinAs.
(2) A real-time PCR, primers BNITMSARS1, BNITMSARAs2, probe BNITMSARP.

All BNI PCRs have been published in an early release article in NEJM on Wednesday, April 10th (http://content.nejm.org/cgi/reprint/NEJMoa030747v2.pdf). The positions of the recommended primers can also be looked up there. Both PCR's allow the detection of single RNA copies and do not cross-react with either human coronaviruses or various animal coronaviruses (mouse hepatitis virus, bovine coronavirus, avian infectious bronchitis virus, porcine coronaviruses).
As a latest development, BNI's real-time PCR has been transformed into a diagnostic kit by **artus-biotech**, a company specialized in providing PCR kits for diagnosic applications.

Role of the BNI: The BNI is an academic institution. BNI has asked two Biotech companies for assistance in distributing necessary test reagents. Company 1 is **TIB MOLBIOL** in Berlin, company 2 is **artus-biotech** in Hamburg. BNI itself distributes free of charge a cloned and in-vitro transcribed 200 bp fragment of the SARSV polymerase gene containing the target region of its SARSV PCRs. The RNA is shipped in lyophilized form to every laboratory interested in establishing BNI's diagnostic tests. So far, >80 laboratories worldwide have obtained the RNA. Contact: drosten@bni-hamburg.de.

Role of **TIB MOLBIOL**, Berlin: TIB MOLBIOL has synthesized all primers and probes developed by the BNI for SARSV in large scale stocks. TIB MOLBIOL synthesizes the primers described in six sites:

Roche Diagnostics **Taiwan**	Kevin.Lee.KL4@Roche.com
Roche Diagnostics **Singapore**	Jane.Lo@Roche.com
Roche Diagnostics **Hong Kong**	John.Tse@Roche.com
TIB MOLBIOL **Berlin**, **Germany**	dna@tib-molbiol.de
TIB MOLBIOL **Freehold, USA**	ethuroff@tibmolbiol.com
TIB MOLBIOL **Genova , Italia**	dna@tibmolbiol.it

In addition to supplying oligonucleotides, TIB MOLBIOL gives application support to labs establishing tests with these primers and probes. Detailed working bench protocols are also provided. Upon request, positive control material (plasmid) can be obtained from TIB MOLBIOL.

Role of **artus-biotech**, Hamburg: Artus has assembled a real-time RT-PCR kit based on BNI's set. The technical performance of the kit has been approved by BNI. The test can be used on different real-time machines, including the Roche LightCylcer and Corbett Rotorgene. It contains an internal control that allows to monitor the presence of PCR inhibitors in each test sample. In light of the situation, artus will distribute alimited number of kits free of charge, to allow laboratories to evaluate this kit. Further information can be obtained from artus project leader Thomas Laue (laue@artus-biotech.de) or via http://www.artus-biotech2.com/en/framesets/index_news.php?id=24.

Please note that the WHO definition of SARS does not include findings in PCR.

Statement of WHO regarding the use of PCR for SARSV: "Results of PCR ... can be used to complement clinical diagnostic evaluation. However, tests have not been validated for confirmation of cases or exclusion of the disease. The WHO case definition remains unchanged at present. Samples from potential SARS patients (suspected or probable) should be performed under conditions of **Biosafety level 3 (BSL-3)**. Biosafety level 3+ is required for viral culture and animal experiments."

Christian Drosten **Bernhard-Nocht Institute for Tropical Medicine**