

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Grant PR, Garson JA, Tedder RS, et al. Detection of SARS Coronavirus in Plasma by Real-Time RT-PCR. *N Engl J Med* 2003;349:2468-9.

Supplementary Appendix 1. Quantitative Detection of SARS Coronavirus by Real-Time RT-PCR and Demonstration of the Viraemia Time Course during the Early Acute Phase

Sars CoV RNA Detection and Quantification Protocols

RNA Extraction

SARS CoV RNA was extracted using the BioRobot 9604 with the QIAamp96 Virus protocol (Qiagen, Hilden, Germany) according to the manufacturer's instructions, except with the first section (viral lysis) performed manually in a safety cabinet. Brome Mosaic virus (BMV) RNA (Promega) was introduced with the lysis buffer and co-extracted with the SARS CoV RNA to act as an internal control. The BioRobot was used to dispense 40 µl of protease to a 96 well block and then paused to allow the block to be removed. In a safety cabinet, 200 µl of EDTA plasma (preferred) or serum sample were added manually to each well of the block. 200 µl of viral lysis buffer (AL) were then added manually to each well and mixed by gentle agitation. The lysis buffer contained 1 µl of a 1 in 10⁶ dilution of BMV RNA per sample (5 pg BMV RNA). The block was then placed onto the BioRobot for the rest of the extraction procedure to be completed as in the standard protocol. Briefly; incubation at 56°C for 10 min followed by addition to 230 µl of 100% ethanol. This mixture was passed through the 96-well QIAamp plate by vacuum aspiration and washed three times. The RNA was eluted from the silica matrix in 86 µl of RNase-free water.

Qualitative Nested RT-PCR (First Round)

The RNA was reverse transcribed and amplified using the Qiagen One Step RT-PCR system. A 50 µl reaction containing 20 µl of the extracted RNA and 30 µl master mix was prepared as follows: 10 µl 5× RT-PCR buffer (as supplied); 10 µl 5× 'Q solution' (as supplied); 2 µl of the dNTP mix (as supplied); 20 pmol each of the SARS CoV primers¹ BNIoutS2 5'-ATG AAT TAC CAA GTC AAT GGT TAC and BNIoutAS 5'-CAT AAC CAG TCG GTA CAG CTA C, 2 µl Qiagen enzyme mix (as supplied) and nuclease-free water to a final volume of 30 µl. The master mix was pipetted into a 96 well PCR plate and 20 µl of the RNA extract was added to each well. The plate was sealed using an adhesive foil seal.

Thermal cycling was performed in a GeneAmp 9700 (Applied Biosystems, Foster City, CA, USA) with the following cycling conditions: 50°C for 30 min for reverse transcription, then 94°C for 15 min to activate the Taq polymerase, followed by 45 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec.

Qualitative Nested RT-PCR (Second Round)

The second round of PCR was performed using Qiagen *Taq* PCR Master Mix as follows: 1 µl of the first round PCR product was transferred to a 24 µl second round mix containing 5 pmol of each of the SARS CoV primers¹ BNIinS 5'-GAA GCT ATT CGT CAC GTT CG and BNIinAS 5'-CTG TAG AAA ATC CTA GCT GGA G. After an initial hold at 94°C for 1 min, 23 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec were performed. The second round products (108 bp) were detected by agarose gel electrophoresis.

Quantitative Nested Real-Time RT-PCR (First Round)

The first round RT-PCR for the quantitative protocol was the same as the first round for the qualitative protocol with the addition of 5 pmol each of the BMV primers BMVseq1 5'-CGG ATC TCT CGC GAG TTT TA and BMVseq2 5'-CAC AGT TCG AGG AAT CAC TG. Twenty cycles of PCR were performed using the same cycling parameters as for the qualitative protocol.

Quantitative Nested Real-Time RT-PCR (Second Round)

The second round PCR was performed in an ABI Prism 7000 sequence detection system (Applied Biosystems). The SARS CoV primers and probe were adapted from those of Drosten et al., 2003.¹ The reaction mix was prepared using 12.5 µl 2× TaqMan universal PCR master mix (Applied Biosystems) with 5 pmol each of SARS CoV primers BNITMSARS1 5'-TTA TCA CCC GCG AAG AAG CT and SARSTAQ2 5'-TCT AGT TGC ATG ACA GCC CTC and 5 pmol each of BMV primers BMVTAQ1 5'-GTT CAC CGA TAG ACC GCT G and BMVTAQ2 5'-AAG AGC CCG GAA TGT CAA GA. The SARS CoV probe (10 pmol) SARSPR 5'-*FAM*-GTT CGT GCG TGG ATT GGC TTT GAT GA-*TAMRA* and the BMV internal control probe (10 pmol) BMVTAQPR 5'-*VIC*-CCT CAA GCT GAA ATG GCA CGG ATG-*TAMRA* were both added to the reaction mix. The total master mix volume of 15 µl per reaction was pipetted into a 96 well optical PCR plate (Applied Biosystems) and 10 µl of the first round product was added to each well. The plate was sealed using an optical adhesive seal (Applied Biosystems).

The reaction was held for 2 min at 50°C followed by 10 min at 95°C to activate the Taq Gold. The reaction was then cycled for 40 rounds of 95°C for 15 sec and 60°C for 1 min. SARS CoV positive samples were quantified by comparison with a standard curve generated from 10 fold dilutions of a SARS CoV RNA transcript of known concentration obtained from Dr. Christian Drosten (Bernhard Nocht Institute, Hamburg, Germany).

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References

1. 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.