Validation of qRT-PCR assays for detection of novel coronavirus 2019-nCoV

5.1.2e 5.1.2e, 5.1.2e 5.1.2e 5.1.2e 5.1.2e, 5.1.2e 5.1.2e 5.1.2e 5.1.2e, 5.1.2e 5.1.2e, 5.1.2e 5.1.2e, 5.1.2e 5.1.2e

National Institute for Public Health and the Environment (RIVM)

Centre for Infectious Diseases Research, Diagnostics and laboratory Surveillance;

Centre for Infectious Disease Control

Background surveillance acute respiratory infections

The clinical specimens for the clinical validation of the E-assay and GdRp-assay were selected from the biobank containing combined nose swab and throat swab specimens in virus transport medium from the national surveillance of influenza-like illness (ILI) and acute respiratory infections (ARI) in the Netherlands. General practitioners from the Nivel Netherlands institute for health services research Primary Care Database report the incidence of ILI on a weekly basis and take nasopharygeal and oropharyngeal swabs from patients consulting with ILI or ARI. These specimens are submitted to the National Institute for Public Health and the Environment (RIVM) for in house developed qRT-PCR diagnosis of influenza virus, respiratory syncytial virus, rhinovirus and enterovirus. When questions arise on unsuspected increases in ILI incidence accompanied by a low detection rate of pathogens the FTD Respiratory pathogens 21 assay (Fast Track Diagnostics) is used.

Selection of specimens

One-hundred specimens have been selected from the surveillance biobank (Table 1). For influenza viruses, RSV and rhinovirus about ten specimens with Ct values below 30 and representative for the range of Ct values in clinical practice. For enterovirus the range of types detected in respiratory surveillance; for most a low and higher Ct value per type. For the other pathogens those detected with a Ct value below 30 during increased ILI incidence at the start of the 2018/2019 respiratory season that could not be fully explained by routinely detected viruses.
Additional were tested, CoV-HKU1 RNA from highly positive specimen, CoV-NL63 virus isolate, CoV-229E virus isolate, *Chlamydia psittaci* culture, *Chamydophila pneumoniae* culture, *Mycoplasma pneumoniae* culture and the 2019 MERS-CoV QCMD EQA panel containing different dilutions of MERS-CoV in 5 specimens, CoV-OC43 (n=1) and CoV-NL63)n=1) virus and 1 negative specimen. Except for CoV-HKU1 RNA all specimens were freshly extracted.

Table 1. Tests of known respiratory viruses and bacteria in clinical specimens

Clinical specimens with known viruses/bacteria	Number of samples tested in both assays	Ct range
CoV-HKU1	1	NA
CoV-OC43	5	22.7 – 29.9
CoV-229E	3	25.1 – 28.5
Influenza virus A(H1N1)pdm09	10	18.9 – 28.1
Influenza virus A(H3N2)	10	17.4 – 27.7
Influenza virus B/Victoria	7	19.8 – 29.9
Influenza virus B/Yamagata	10	23.5 – 29.1
Rhinovirus	13	13.4 – 28.0
Enterovirus (for types see supplementary excel file)	18	19.6 – 28.9
Respiratory syncytial virus A	10	18.0 – 29.8
Respiratory syncytial virus B	11	21.0 – 29.4
Parainfluenza virus 3	1	26
Human metapneumovirus	3	26.7 – 27.6
Adenovirus	2	21.0 – 27.3
Human Bocavirus	1	35 (borderline)
Mycoplasma spp.	1	26.8
Total	106	

qRT-PCR

Per clinical specimen 200 µl was extracted on a MagNA Pure 96 Instrument (Roche) using the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche) and eluted in a volume of 50 µl. E-assay and RdRP-assay primers and probes as described in the provided protocol by Corman et al. were kindly provided by Corman and ordered from Eurogentec (Belgium). As control material SARS full virus RNA (Strain Frankfurt 1) (Ct value 29-30 by 5 µl/rx; 10e3 genome copies/µ) was also kindly provided by Corman.

Reaction conditions were as follows (Table 2, 3):

Table 2. For primers and probes provided by Corman.

E-gene qRT PCR	µl	RdRP-gene qRT-PCR	µl
4x Taqman Fast Virus MM	5	4x Taqman Fast Virus MM	5
SJVE-F (10 µM)	1	SJVR-F (10 µM)	1.5
SJVE-R (10 µM)	1	SJVR-R (10 µM)	2.0
SJVE-P (10 µM)	0.5	SJVR-P1/SJVR-P2 (10 µM)	0.5
PCR grade water	7.5	PCR grade water	6
Specimen nucleic acid	5	Specimen nucleic acid	5
Total volume	20 µl	Total volume	20 µl

Table 3. For primers and probes obtained from Eurogentec primers and probes were premixed at a final concentration of 10 µM each except for the SJVR probes for 5 µM each.

E-gene qRT PCR	µl	RdRP-gene qRT-PCR	µl
4x Taqman Fast Virus MM	5	4x Taqman Fast Virus MM	5
SJVE Mix (10 µM)	3	SJVR Mix (10 µM)	3
PCR grade water	7	PCR grade water	7
Specimen nucleic acid	5	Specimen nucleic acid	5
Total volume	20 µl	Total volume	20 µl

Table 4. Amplification temperature protocol.

PCR Program	Segment number	Temp Target (°C)	Hold Time (sec.)	Slope (°C/sec.)	Acquisition mode	
Reverse Transcription	**1**	**50**	**900**	**EXTERNAL**		
Denaturation/Inactivation	**1**	**95**	**120**	**EXTERNAL**		
Denaturation	1	95	60	4.4	None	LC 480
Amplification (cycles:50)	1	95	10	4.4	None	
	2	58	30	2.2	Single	
Cooling	1	40	30	4.4	None	

Standard EAV external control qRT-PCR was performed to control for inhibitors.

LOD95 determination

10-fold dilution series of control material were amplified using the reference primers and probes and the Eurogentec ordered primers and probes. Based on this dilution series a starting point for 2-fold dilution series in 4-fold was chosen, prepared and subjected to amplification to determine the LOD95.

Table 5. 10-fold dilution series both primer and probe sets

Name	E-gene		RdRP-gene	
Dilution	Eurogentec (EuroE) Ct	Duitsland (DuitE) Ct	Eurogentec (EuroR) Ct	Duitsland (DuitR) Ct
SARS undiluted	26.09	25.15	27.08	28.77
SARS 10-1	29.18	28.32	30.49	31.48
SARS 10-2	31.84	30.85	33.23	32.98
SARS 10-3	33.99	34.01	35.68	34.5
SARS 10-4	neg	35.92	35.75	34.97
SARS 10-5	neg	36.12	neg	neg
SARS 10-6	neg	35.18	neg	neg
SARS 10-7	neg	35.26	neg	neg
Neg extraction control 1	neg	34.44	neg	neg
Neg extraction control 2	neg	ND	neg	neg

nd = not done

Eurogentec E-gene primers and probe give a cleaner picture (Figure 1). German E-gene primers and probe generate 'ghost' curves in high dilutions and negative control (Figure 1). For the RdRP-gene qRt-PCR primers and probes of both sources the qRT-PCRs generated similar results. For both qRT-PCR assays the Eurogentec primers and probes generated higher fluorescence values than the German primers and probes.

Figure 1. Amplification curve E-gene qRT-PCR

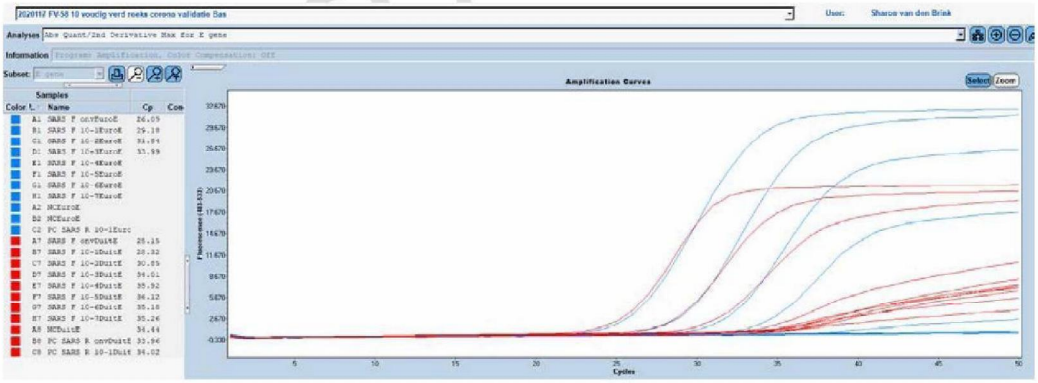


Figure 2. Amplification curve RdRP-gene qRT-PCR

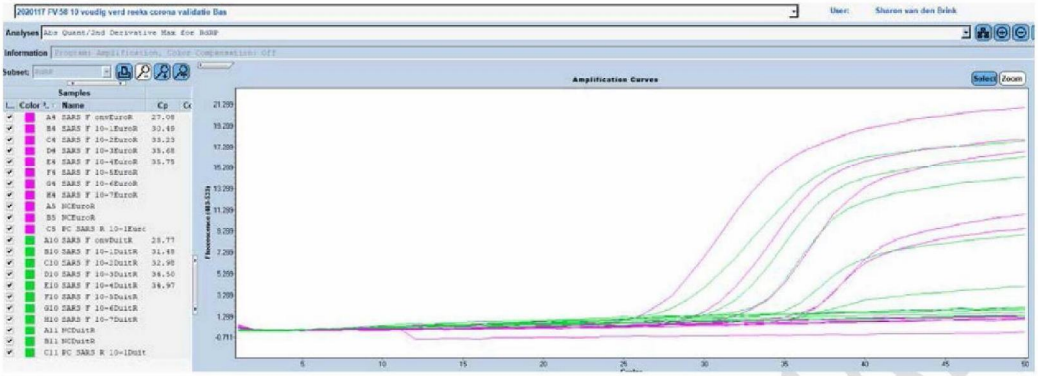
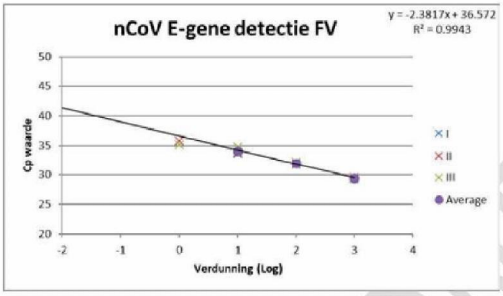
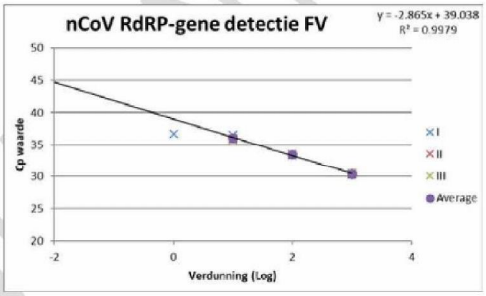


Figure 3. Linearity and amplification efficiency Eurogentec primers and probes





Based on the 10-fold dilution series the efficiency was calculated:

	Slope	$E = 10^{-1/slope}$	$E\ (\%) = 100 \times [10^{-1/slope} - 1]$	R^2
Theoretical	**-3.322**	**2.00**	**100**	**0.9500**
nCoV E-gene	-2.382	2.63	162.95	0.9943
nCov RdRP-gene	-2.865	2.23	123.38	0.9979

Efficiency is excellent.

Using the 2-fold dilution series repeated in 4-fold the LOD95 was calculated (Eurogentec primers and probes):

qRT-PCR	E-Gene	RdRP-Gene
	Copies per µl	Copies per µl
LOD 95%	6.362E-01	7.421E-01
lower bound	4.388E-01	5.626E-01
upper bound	1.363E+00	1.595E+00

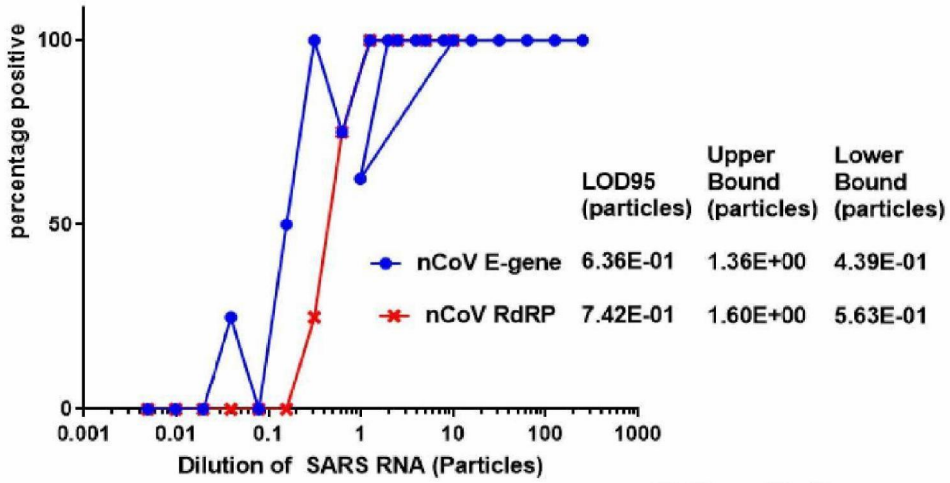


Figure 4. 95% limit of detection curves with calculated LOD95; Eurogentec primers and probes

Clinical specimens

Except for two of the clinical specimens and other cultured viruses, the cultured bacteria and the 2019 MERS-CoV QCMD EQA panel specimens were negative in the qRT-PCR with Eurogentec primers and probes.

One (with Ct 28.1) of 10 influenza A(H1N1)pdm09 positive specimens and one (with Ct 29.3) of 11 RSV-B positive specimens were initially positive in the E-gene qRT-PCR only with high Ct values of 37.7 and 39.1 respectively (Figure 5). Both specimens have been repeated in the E-gene RT-PCR with annealing temperatures of 58°C and 60°C and turned out negative. None of the other A(H1N1)pdm09 and RSV-B positive specimens with higher viral load generated a positive signal in the E-gene qRT-PCR.

Figure 5. Amplification curves of clinical specimens with E-gene qRT-PCR