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Revalidation Report **Detection of SARS-Cov2 RNA by RT-PCR**	Validation ID: APG20_020 Version: 1.0

Review / Approval

	Name	Date (DD.MM.YYYY)	Signature
Author 5.1.2e			
Technical Review/Approval 5.1.2e	5.1.2e		
QM/QA Review 5.1.2e			
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Approval Management 5.1.2e			

1 Introduction

The aim of this revalidation was to validate the modifications of Eurofins inhouse RT-PCR assay (internal validation ID: APG20_015) for qualitative detection of the novel coronavirus (SARS-CoV-2) RNA from human respiratory samples due to the change of the 1. oligos/probes supplier (from ELLA Biotech GmbH to Eurofins Genomics Europe Synthesis GmbH), 2. IPC molecule type (switch from DNA to RNA) and 3. fluorescence dye of the E-gene assay.

2 Scope and limitations of revalidation

The revalidation was intended to check if the following changes of the validated SARS-CoV2 inhouse assay (APG_2020-003) have any impact on the performance of the validated inhouse SARS-CoV2 assay:

- Change 1: For the in-house SARS-CoV2 assay, oligos/probes from Eurofins Genomics Europe Synthesis GmbH shall be used and not from external manufacturers as before.

- Change 2: For the internal positive control (HPV target) synthetic RNA will be used instead of plasmid DNA

- Change 3: For the E-gene assay (Sarbeco Assay) a 5'-Atto647N labeled sample should be used instead of a 5'-FAM labeled sample.

Since all other assay parameters were unchanged only those criteria were evaluated, which probably may be affected by potentially different oligo/probe quality. Assuming that oligos/probes(here in particular fluorescence dye with lower quality) could contain residues (from oligo synthesis) or contaminations, which cause lower PCR efficiency or higher background signal, the sensitivity and, as a consequence, the limit of detection of the assay will probably mostly influenced by a potential difference in oligo/probe quality. Moreover the sensitivity again influences the accuracy with which low target number can be detected and identified. Additionally precision wias tested in order to ensure reproducibility.

3 Summary

The following table concludes the results of the setup experiments:

Validation Parameter	Results	Acceptance criteria	Status
Specificity	100%	As close to 100% as possible	n.a.(since no specific criterion was defined in the validation plan
Sensitivity	96%	As close to 100% as possible	n.a.(since no specific criterion was defined in

			the validation plan
Precision	SD <0,5 (repeatability); SD < 1,0 (intermediate precision)	SD <0,5 (repeatability); SD < 1,0 (intermediate precision)	passed
LOD	1 copy RNA/RT-PCR	As low as possible	n.a.(since no specific criterion was defined in the validation plan
Accuracy	98,7%	As close to 100% as possible	n.a.(since no specific criterion was defined in the validation plan

4 Definitions and abbreviations

Term / Abbreviation	Definition
APG	Applied Genomics
DNA	deoxyribonucleic acid
cDNA	complementary DNA
dPCR	digital PCR
IPC	Internal positive control
LOD	Limit of Detection
LOQ	Limit of Quantification
NTC	No Template Control
RT-PCR	real-time PCR
RNA	ribonucleic acid
qPCR	quantitative Polymerase Chain Reaction

5 Responsibilities

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6 Method

6.1 Description of the procedure

The detection was performed in one-step real-time RT-PCR format, i.e. reverse transcription (RT) and the subsequent PCR took place in a reaction vessel. The isolated RNA was transcribed into cDNA. The specific gene fragments for SARS-CoV-2 (N1-gene, E-gene) were subsequently amplified by real-time PCR. The amplified target sequences were treated with hydrolysis probes, which are connected at one end to a quencher and at marked at the other end with a reporter fluorescent dye (fluorophore). In the presence of a target sequence, the probes hybridize with amplicons. During the extension, the Taq polymerase separates the reporter from quencher. The reporter emits a fluorescent signal which is detected by the optical unit of a real-time PCR device is detected. The fluorescence signal increases with the amount of amplicons formed. The assays contained an Internal Control RNA or Plasmid (ICR/ICP) and corresponding primers/probe to facilitate sample preparation and/or control potential PCR inhibition.

6.1.1 RNA Extraction

The RNA extraction was performed on the KingFisher extraction system by using the PurePrep Pathogens Kit from Molg3n (Utrecht, Netherlands; Art.No.OE00290096). Positive and negative extraction controls (included within the kit) were included in every extraction run.

6.1.2 Digital droplet PCR (dPCR)

The digital PCR was used in order to quantify the reference RNA, which was used as positive control. The RNA was reverse transcribed and amplified by digital polymerase chain reaction (dPCR). The dPCRs was performed using the N1-gen assay in a reaction volume of 25 µl. For primer/probe assay *qScript XLT One-Step RT-qPCR ToughMix* (95132) was used.

General characteristics	
Capacity	Up to 4 samples/chip
Input volume	Up to 25 µL/sample
Droplets per sample	25 000 - 30 000
Droplet volume	0.59 nL
Dynamic range	5 logs (0.2 - 15 700 cp/µL)
Minimum quantification error at 95% confidence level	1.5%

Figure 1 General characteristics of the STILLA Naica system (copied from the UserManual Naica Crystal Digital PCR System v2.0)

The ddPCR setup and program are described in the tables below. The analysis was performed with the STILLA Naica System instrument, which is capable of analyzing up to 12 samples on 3 sapphire chips per run.

Table 1 dPCR set-up for primer/probe-mastermix

Component	Final Conc.	µl
qScript XLT 1-Step RT-qPCR ToughMix (2x)	1x	12,500
Fluorescein (1 µM)	100 nM	2,500
2019-nCoV_N1-F (100 µM)	1.0 µM	0,25
2019-nCoV_N1-R (100 µM)	1.0 µM	0,25
2019-nCoV_N1-P (100 µM)	0.25 µM	0,0625
RNA	-	4.00
H2O	-	4,875
Final Volume (µl)	-	25.00

Table 2 ddPCR program for primer/probe setup

Temperature [°C]	Duration	Cycles
50	10	1x
95	1 min	1x
95	10 sec	45x
60	30 sec	

6.1.3 RT-PCR

The sample RNA extracts and the reference RNA (Twist Synthetic SARS-CoV2 RNA Control) were used as template and were transcribed in cDNA and amplified by quantitative polymerase chain reaction (qPCR) in a two-step reaction (see table 1). The PCR reaction was run with as triplex PCR consisting of two SARS-CoV2 specific assays (SARS-CoV E-gene and for 2019-nCoV_N1 assay) and one control assay ("HPV assay"). The reference RNA was set into RT-PCR in a LOG10 dilution series (V1-V5 with V1 = 1,00 +E05 RNA copies/µl). Additionally, a reference plasmid was be set into RT-PCR in a LOG10 dilution series (V1-V10 with V1 = 1,00 +E09 DNA copies/µl). The RT-PCRs were performed in a reaction volume of 12 µl with *2x qPCRBio Probe 1-Step Go Mix (Lot-No. PB092618-032-5).* The PCR primers/probe sequences are displayed in 6.2.2.1

The real time qPCR setup and program are described in tables 3 and 4. The analysis was performed with the Lightcycler480II instrument in 384-well format.

Table 3: Mastermix for single step RT-PCR (reverse transcription+qPCR)

Component	Volume (µl)
2x qPCRBIO Probe 1-Step Go Mix	6,000
Promega H_2O	0,696
RNA_IC_AmpTec (10^6 cop./µL)	0,03
ic_fw1 (100µM)	0,048
ic_bw1 (100µM)	0,048
ic_p1r (HEX) (100µM)	0,029
2019-nCoV_N1-F (100µM)	0,048
2019-nCoV_N1-R (100µM)	0,048
2019-nCoV_N1-P (100µM)	0,029
E_Sarbeco_F1 (100µM)	0,048
E_Sarbeco_R2 (100µM)	0,048
E_Sarbeco_P (100µM)	0,029
20x Rtase Go	0,900
Eluat (RNA)	4,000

Table 4: Cycling conditions for RT-PCR

Step	°C	Time	No. of Cycles
pre-Incubation	50	2 min	1
Initial denaturation	95	10 min	1
Denaturation	95	15 sec	45
Annealing/Elongation	60	1 min	45
Cooling	4	∞	-

6.2 Equipment and Material

6.2.1 Equipment

Description	Supplier
Eppendorf Research® plus (single channel pipette) (100-1000 µl/ 20-200 µl/ 10-100 µl/ 2-20 µl/ 0,5-10 µl/ 0,1-2,5 µl) (or comparable instrument)	Eppendorf AG, Hamburg, Germany
PIPETMAN® Classic (P2, P10, P20, P100, P200, P1000) (or comparable instrument)	Gilson S.A.S., Villiers-le-Bel, France
Centrifuge MiniSpin® plus (or comparable instrument)	Eppendorf AG, Hamburg, Germany
Centrifuge 5810 R (or comparable instrument)	Eppendorf AG, Hamburg, Germany
Vortex-shaker (or comparable instrument)	VWR International GmbH, Darmstadt, Germany
LightCycler® 480 Instrument II	Roche Diagnostics GmbH, Mannheim, Germany
STILLA, Naica system (Prism and Geode)	STILLA Technologies, Villejuif, Frankereich
NanoDrop Lite®	Thermo Scientific, Schwerte, Deutschland
Trinean Dropsense96®, Photometer	Trinean, Gentbrugge, Belgium

Note 1: All equipment used in this validation was qualified for the application, released and functional. Test and measuring equipment was calibrated, calibrations were valid.

6.2.2 Materials

6.2.2.1 Primer/Probes

Primer and probe sequences for the SARS-CoV E-gene and for 2019-nCoV_N1 assay are taken from the WHO recommendations. The probe for the N1-assay is FAM-labelled, whereas the probe for the E-gene will be tested with FAM (old design) as well as with Atto647N (new design). The probe for the HPV target is HEX-labelled, in order to be able to perform duplex or triplex PCR reactions.

2019-nCoV_N1 assay:

2019-nCoV_N1-F:	5`-GAC CCC AAA ATC AGC GAA AT-3`
2019-nCoV_N1-R:	5`-TCT GGT TAC TGC CAG TTG AAT CTG-3`

2019-nCoV_N1-P: 5`- FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3`

5`>GACCCCAAAATCAGCGAAATGCACCCCGCATTACGTTTGGTGGACCCTCAGATTCAACTGGCAGTAACCAGA<3`

Amplicon size: 72 bp

E-gene assay:

E_Sarbeco_F1:	5`-ACAGGTACGTTAATAGTTAATAGCGT -3`
E_Sarbeco_R2:	5`- ATATTGCAGCAGTACGCACACA-3`
E_Sarbeco_P1:	5`-FAM- ACACTAGCCATCCTTACTGCGCTTCG-BHQ1-3´

5`>ACAGGTACGTTAATAGTTAATAGCGTACTTCTTTTTCTTGCTTTCGTGGTATTCTTGCTAGTCACACTAGCCATCCTTACTGCGCTTCGATTGTGTGCGTACTGCTGCAATAT<3`

Amplicon size: 113 bp

Mycoplasma assay:

ic_fwd1:	5`- GAATTGTGTGCCCCATCTGTT-3`
ic_bw1:	5`- TCCGGTGTCTGGCTCTGATCTT -3`
ic_p1r:	5`- BBQ- GATCCTGCAGCAGCAACGAAGTATCCTCT -HEX -3´

5`>GGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCAATTGGGTACCGAGCTCGCGGCCGCAAGCCCTGTTAATGGGCACACTAGGAATTGTGTGCCCCATCTGTTCTCAGAAACCATAATCTACCATGGCTGATCCTGCAGCAGCAACGAAGTATCCTCTCCTGAAATTATTAGGCAGCACTTGGCCAACCACCCCGCCGCGACCCATACCAAAGCCGTCGCCTTGGGCACCGAAGAAACACAGACGACTATCCAGCGACCAAGATCAGAGCCAGACACCGGAAACCCCTGCCACACCACTAAGTTGTTGCACAGAGACTCAGTGGACAGTGCTCCAATCCTCACTGCATTTAACAGCTCACACAAAGGACGGATTAACTGTA <3` Amplicon size: 199 bp

6.2.2.2 Sample Material

Any additional samples used for this validation study are listed in the following table

Description	Supplier	Validation Parameter
Clinical routine samples, tested with Eurofins in-house kit (containing ELLA oligos/probes)	Eurofins Genomics Europe Applied Genomics GmbH, Ebersberg, Germany	- Accuracy - Precision - Specificity - Sensitivity

6.2.3 Reagent and kits

Description	Storage	Supplier
Nuclease-Free Water	Room temperature	Promega GmbH, Mannheim, Germany
qPCRBIO Probe 1-Step Go No-ROX	-30°C to -15°C (12 month)	PCR Biosystems Limited, London, UK (Cat. No.: PB25.43)
qScript XLT One-Step RT-qPCR ToughMix (95132)	-25°C to -15°C	Quantabio, Beverly, USA

Note 2: All materials, kits and reagents as outlined in 6.2.2 and 6.2.3 used were approved for use. Expiration dates were not exceeded. Kits and reagents were stored according to the manufacturer's instructions in monitored facilities.

6.3 Reference Materials

The reference materials to be used in this validation are listed in the next table. All concentrations of the various standards are based on dilutions of the respective primary standard (PRI).

Reference material	Supplier	Validation Parameter
Synthetic RNA control for variant of the SARS-CoV-2 virus: MT007544.1 (GenBank IDs).	Twist Bioscience, San Francisco, USA (Catalog No 102019)	LOD
Synthetic RNA used as internal positive control, named "ssRNA MYCOPOSKO"	AmpTec GmbH, Hamburg, Germany	n.a.

7 Results

7.1 RNA Quantification via digital PCR

4 µl of reference RNA (Cat. No. 102019, Twist Bioscience) with concentrations of 1000 copies/µl, 100 copies/µl and 10 copies/µl (concentration data is based on the manufacturer`s specifications) was set into 25 µl dPCR reactions and analyzed only with the N1-gene assay since the dPCR is based on endpoint PCR.
The FAM signal were detected with a exposure time 40ms.

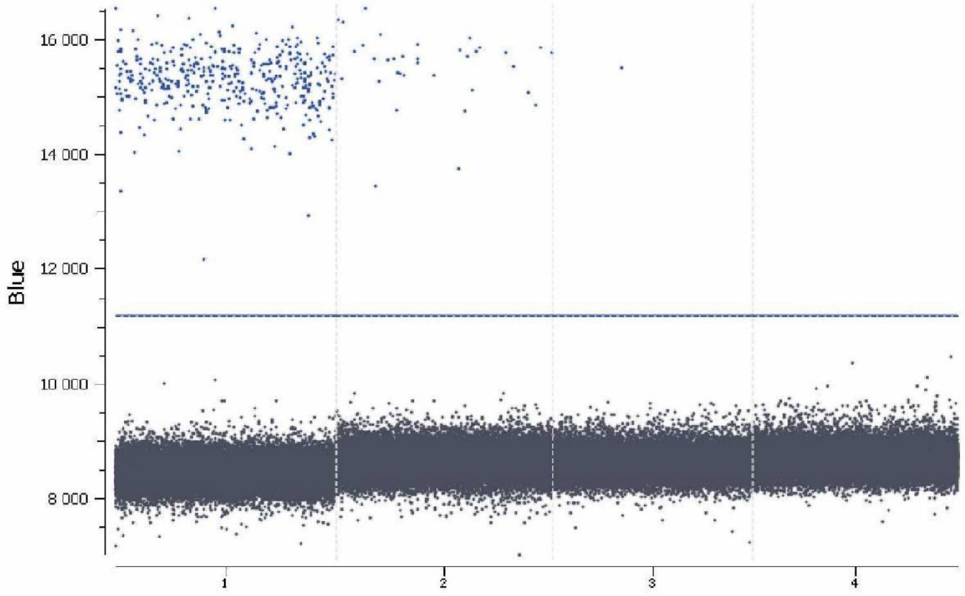
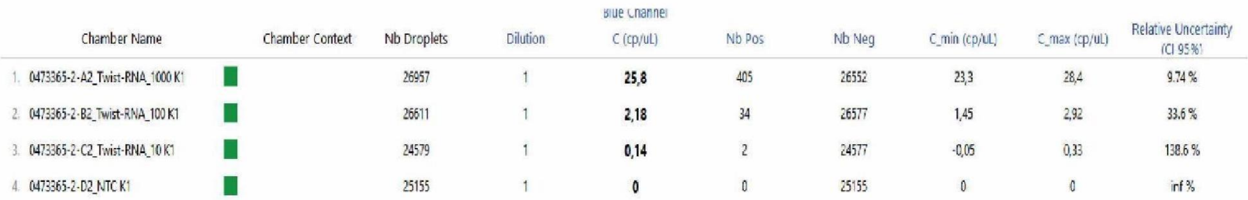


Figure 2: 1D dot plot of positive (blue) and negative (grey) droplets detected in the FAM channel. The dPCR was performed with N1-gene assay three reference RNA dilutions. 1 = 4000 copies/dPCR; 2 = 400 copies/dPCR; 3 = 40 copies/dPCR; 4 = NTC; The threshold line divides the negative from the positive droplets and was set to 11,178 fluorescent units for FAM. ; Y-axis = fluorescence units; X-axis = droplet index.

The concentration results calculated by the Naica software are displayed in the following figure:



	Chamber Name		Chamber Context	Nb Droplets	Dilution	Blue Channel C (cp/uL)	Nb Pos	Nb Neg	C_min (cp/uL)	C_max (cp/uL)	Relative Uncertainty (CI 95%)
1.	0473365-2-A2_Twist-RNA_1000 K1	🟩		26957	1	**25,8**	405	26552	23,3	28,4	9.74 %
2.	0473365-2-B2_Twist-RNA_100 K1	🟩		26611	1	**2,18**	34	26577	1,45	2,92	33.6 %
3.	0473365-2-C2_Twist-RNA_10 K1	🟩		24579	1	**0,14**	2	24577	-0,05	0,33	138.6 %
4.	0473365-2-D2_NTC K1	🟩		25155	1	**0**	0	25155	0	0	inf %

Figure 3: Result table of the RNA copy number calculations. The tbles displayes the chamber name, der number of total droplets, the dilution factor, the measured/calculated concetration, the number of positive droplets, the number of negative droplets, the minima and maxima of the measured concentration and the relative uncertainty for 95% confidence interval.

Since the relative uncertainty is lowest in the results of chamber 1 (1000 copies/µl RNA = 4000 copies/dPCR), the corresponding dilution was used to calculated the "real" RNA concentration. The expected results (based on the manufacturers specifications) was 4µl x 1000 copies/µl RNA = 4000 copies/dPCR, what corresponds to a concentration of 4000/25 = 160 copies/µl dPCR mix. However the measured concentration was 25,8 copies/µl dPCR mix, which reflects a discrepancy of factor 6,2 between stated concentration and measured concentration.

As a consequence the reference RNA for the LOD measurement was adjusted and diluted in alignment with the dPCR measurement, i.e. the stock solution, which has a stated concentration of 1,00E +06 copies/µl RNA (according to the manufacturer`s specifications), was considered to have a real concentration of 1,6E +05 copies/µl RNA and all subsequent RNA concentrations were prepared base on this value.

7.2 Specificity/Sensitivity

7.2.1 Methodology and samples:

The evaluation of sensitivity and specificity was based on the following sample size:

- RT-PCR on 24 positive samples in duplicates
- RT-PCR on 48 negative samples in duplicates

Due to practical reason all samples were analyzed in two repetitions, but only repetition 1 was used for evaluation of specificity and sensitivity. The sample material was taken from routine analysis plates *GE20_0318* and GE20_0322 (internal plate number).

The results of the validation experiments were categorized based on the definitions made in validation report "APG20_015_SARS-Cov2_Report_v4" (signed on 28.04.2020), .i.e. samples with Cp < 38 were considered as “positive”, samples with a Cp value between 33 and 38 were considered as l”ow positive”, whereas signals above Cp 38 were considered as “invalid”. Samples with no signal were counted as “negative”.

Positive controls (plasmid and reference RNA) and NTCs were analyzed together with the sample on every RT-PCR plate.

7.2.2 Test and Results:

The rating of the sample results was based on the following rules:

- A signal with Cp < 33 is rated as “positive”
- A signal with Cp between 33 abd 38 is rated as “ low positive”
- A signal with Cp > 38 is rated as “invalid”
- No signals or unspecific signals are rated as “negative”

The interpretation of the duplicate results was according to following scheme:

		Cp2		
		positive	**low positive**	**negative**
Cp1	**positive**	positive	positive	positve
	low positive	positive	low positive	low positive
	negative	positive	low positive	negative

The interpretation of the test assay and the different channel signals was according to following scheme:

		E-gene Assay		
		positive	**low positive**	**negative**
N1-Assay	**positive**	positive	positive	positve
	low positive	positive	low positive	low positive
	negative	positive	low positive	negative

The following table shows the results obtained with the validated inhouse assay (= reference) as well as the sample results obtained with the test assay in duplicates derived from one repetition (qPCR plate name: PGX20_P0415 and PGX20_P0416). The data of each repetition is shown in attachment 1-4.

Table 5: Comparison of sample results derived from the validated inhouse assay and from the test assay. . The table displays the sample ID, the Cp values of the duplicates (Cp1, Cp2) and the rating of the results (green = positive; orange = low positive; red = negative). Regarding the test assay the table displays the Cp values of duplicates for the FAM and the Cy5 channel, the rating of the results for each single assay and the total rating of the test assay.

	validated inhouse Assay (Repetition 1)			Test Assay (repetition 1)						
	FAM (N1-Assay + E-gene Assay)			FAM (N1-Assay)			Cy5 (E-gene Assay)			
SampleID	Cp1	Cp2	SARS-CoV-2 rating	Cp1	Cp2	SARS-CoV-2 rating	Cp1	Cp2	SARS-CoV-2 rating	total rating
GB00007638	-	-	neg	-	-	neg	-	-	neg	neg
GB00008816	33,61	33,97	low pos	35,62	39,15	low pos	-	-	neg	low pos
GB00010626	-	-	neg	-	-	neg	-	-	neg	neg
GB00010634	-	-	neg	-	-	neg	-	-	neg	neg
GB00010654	-	-	neg	-	-	neg	-	-	neg	neg
GB00010736	28,52	28,06	pos	29,03	29,53	pos	27,68	27,88	pos	pos
GB00010741	-	-	neg	-	-	neg	-	-	neg	neg
GB00010744	-	-	neg	-	-	neg	-	-	neg	neg
GB00010745	-	-	neg	-	-	neg	-	-	neg	neg
GB00010748	-	-	neg	-	-	neg	-	-	neg	neg
GB00010749	27,84	27,61	pos	28,46	28,41	pos	27,06	26,98	pos	pos
GB00010768	-	-	neg	-	-	neg	-	-	neg	neg
GB00010774	23,24	23,31	pos	24,63	24,08	pos	23,59	22,98	pos	pos
GB00010775	-	-	neg	-	-	neg	-	-	neg	neg
GB00010778	-	-	neg	-	-	neg	-	-	neg	neg
GB00010779	24,86	25	pos	25,95	26,52	pos	24,35	24,79	pos	pos
GB00010802	22,93	22,84	pos	23,46	22,85	pos	22,1	21,62	pos	pos
GB00010816	-	-	neg	-	-	neg	-	-	neg	neg
GB00010819	27,83	28,16	pos	28,37	27,88	pos	27,19	26,77	pos	pos
GB00010821	-	-	neg	-	-	neg	-	-	neg	neg
GB00010822	-	-	neg	-	-	neg	-	-	neg	neg
GB00010823	-	-	neg	-	-	neg	-	-	neg	neg
GB00014809	20,71	21,06	pos	21,19	21,1	pos	20,58	20,49	pos	pos
GB00014812	-	-	neg	-	-	neg	-	-	neg	neg
GB00015711	34,98	-	low pos	40,28	43,75	invalid	-	-	neg	invalid
GB00015772	-	-	neg	-	-	neg	-	-	neg	neg
GB00015774	37,16	35,78	low pos	36,74	36,86	low pos	-	-	neg	low pos
GB00016014	-	-	neg	-	-	neg	-	-	neg	neg
GB00016075	-	-	neg	-	-	neg	-	-	neg	neg
GB00016076	-	-	neg	-	-	neg	-	-	neg	neg

GB00016083	-	-	neg	-	-	neg	-	-	neg	neg
GB00016147	-	34,1	low pos	-	-	neg	-	-	neg	neg
GB00016289	29,86	30,04	pos	30,08	30,6	pos	26,48	26,05	pos	pos
GB00016290	27,11	27,13	pos	26,54	26,47	pos	24,59	24,35	pos	pos
GB00016471	22,04	22,14	pos	23,05	22,98	pos	20,72	20,73	pos	pos
GB00021150	-	-	neg		38,14	low pos	-	-	neg	low pos
GB00021151	19,78	19,58	pos	20,49	20,29	pos	18,34	18,22	pos	pos
GB00021155	-	-	neg	-	-	neg	-	-	neg	neg
GB00022865	-	-	neg	-	-	neg	-	-	neg	neg
GB00022875	-	-	neg	-	-	neg	-	-	neg	neg
GB00022879	28,47	28,4	pos	28,25	28,46	pos	27,73	27,81	pos	pos
GB00022881	30,69	30,03	pos	31,1	31,59	pos	28,89	28,95	pos	pos
GB00022882	-	-	neg	-	-	neg	-	-	neg	neg
GB00022886	-	-	neg	-	-	neg	-	-	neg	neg
GB00022904	-	-	neg	-	-	neg	-	-	neg	neg
GB00022911	-	-	neg	-	-	neg	-	-	neg	neg
GB00022912	-	-	neg	-	-	neg	-	-	neg	neg
GB00022917	-	-	neg	-	-	neg	-	-	neg	neg
GB00022936	-	-	neg	-	-	neg	-	-	neg	neg
GB00022945	-	-	neg	-	-	neg	-	-	neg	neg
GB00022947	-	-	neg	-	-	neg	-	-	neg	neg
GB00022951	43,43	-	invalid	-	-	neg	-	-	neg	neg
GB00022955	-	-	neg	-	-	neg	-	-	neg	neg
GB00022969	-	-	neg	-	-	neg	-	-	neg	neg
GB00022971	-	-	neg	-	-	neg	-	-	neg	neg
GB00023070	31,5	31,25	pos	31,76	32,79	pos	29,5	29,73	pos	pos
GB00023079	-	-	neg	-	-	neg	-	-	neg	neg
GB00023156	-	-	neg	-	-	neg	-	-	neg	neg
GB00023181	-	-	neg	-	-	neg	-	-	neg	neg
GB00023901	-	35,45	low pos	38,26	38,09	low pos	-	-	neg	low pos
GB00023913	26,15	26,26	pos	26,06	25,66	pos	24,03	23,48	pos	pos
GB00023914	-	-	neg	-	-	neg	-	-	neg	neg
GB00023919	-	-	neg	-	-	neg	-	-	neg	neg
GB00023975	34,39	33,53	low pos	34,59	37,76	low pos	-	-	neg	low pos
GB00023977	-	-	neg	-	-	neg	-	-	neg	neg
GB00023980	-	-	neg	-	-	neg	-	-	neg	neg
GB00024138	33,68	33,85	low pos	34,77	34,48	low pos	-	-	neg	low pos
GB00024278	-	-	neg	-	-	neg	-	-	neg	neg
GB00024279	25,14	25,26	pos	24,79	24,57	pos	23,7	23,45	pos	pos

GB00024282	-	-	neg	-	-	neg	-	-	neg	neg
GB00024285	-	-	neg	-	-	neg	-	-	neg	neg
GB00024289	32,3	32,51	pos	32,47	33,21	pos	-	28,27	pos	pos
GB00026034	-	-	neg	-	-	neg	-	-	neg	neg

As shown in table 5 the test assay detected 23 out of 24 positive samples (consisting of 17 positive and 7 low positive samples) as “positive”, which corresponds to a sensitivity of (23*100)/24 = 96%. One sample (No. GB00015711) obtained specific signals in the test assay, but the signals were above the LOD range and thus considered as “invalid”. One low positive sample (No. GB00016147) obtained no signal in the test assay and was determined as “negative”.

All of the 48 negative samples showed no signal in the test assay, which corresponds to a specificity of 100%. Interestingly one negative sample (No. GB00021150) was rated as “low positive” in the test assay.

7.3 Limit of Detection

7.3.1 Methodology and samples:

The LOD of the duplex assay was determined by performing RT-PCRs on a 10-fold dilution series of synthetic reference RNA (1,00 E+05 copies/RT-PCR down to 1,00 copiy/RT-PCR) in tduplicates and in two consecutive repetitions.

7.3.2 Test and Results:

Table 6: Results of the LOD evaluation of each repetition and the combined data of all two repetitions detected in the FAM channel (N1-assay). The tables display the dilution steps of the reference RNA, the RNA copy number concentration inserted into RT-PCR, the Cp values of each triplicate, the mean Cp value of the duplicates and the standard deviation of the duplicates. The combined table displays the mean Cp values of each repetition and the mean Cp and SD of all two repetitions. Hyphens indicate, that no signal were detected.

FAM (N1-Assay)						
	Dilution Step	**copies/RT-PCR**	**Cp1**	**Cp2**	**ØCp**	**SD**
Rep. 1	V1	100000	22,53	22,64	22,6	0,1
	V2	10000	25,76	25,77	25,8	0,0
	V3	1000	28,77	28,83	28,8	0,0
	V4	100	32,24	31,81	32,0	0,3
	V5	10	35,15	34,83	35,0	0,2
	V6	1	35,65	-	35,7	-

	Dilution Step	copies/RT-PCR	Cp1	Cp2	ØCp	SD
Rep. 2	V1	100000	21,93	21,91	21,9	0,0
	V2	10000	25,23	25,25	25,2	0,0
	V3	1000	28,34	28,36	28,4	0,0
	V4	100	31,21	31,45	31,3	0,2
	V5	10	33,52	34,84	34,2	0,9
	V6	1	36,49	-	-	-

	Dilution Step	copies/RT-PCR	Rep. 1	Rep. 2	ØCp	SD
Combined	V1	100000	22,6	21,9	22,3	0,5
	V2	10000	25,8	25,2	25,5	0,4
	V3	1000	28,8	28,4	28,6	0,3
	V4	100	32,0	31,3	31,7	0,5
	V5	10	35,0	34,2	34,6	0,6
	V6	1	35,65	-	35,65	-

Table 7: Results of the LOD evaluation of each repetition and the combined data of all two repetitions detected in the Cy% channel (E-gene-assay). The tables display the dilution steps of the reference RNA, the RNA copy number concentration inserted into RT-PCR, the Cp values of each triplicate, the mean Cp value of the duplicates and the standard deviation of the duplicates. The combined table displays the mean Cp values of each repetition and the mean Cp and SD of all two repetitions. Hyphens indicate, that no signal were detected.

	Cy5 (E-gene-Assay)					
	Dilution Step	copies/RT-PCR	Cp1	Cp2	ØCp	SD
Rep. 1	V1	100000	20,79	20,93	20,9	0,1
	V2	10000	23,83	23,98	23,9	0,1
	V3	1000	26,6	26,68	26,6	0,1
	V4	100	28,63	28,56	28,6	0,0
	V5	10	28,95	-	29,0	-
	V6	1	27,75	-	27,8	-

	Dilution Step	copies/RT-PCR	Cp1	Cp2	ØCp	SD
Rep. 2	V1	100000	20,02	20,12	20,1	0,1
	V2	10000	23,23	23,26	23,2	0,0
	V3	1000	26,11	26,2	26,2	0,1
	V4	100	28,63	28,75	28,7	0,1
	V5	10	29,4	30,5	30,0	0,8
	V6	1	-	27,83	-	-

	Dilution Step	copies/RT-PCR	Rep. 1	Rep. 2	ØCp	SD
Combined	V1	100000	20,9	20,1	20,5	0,6
	V2	10000	23,9	23,2	23,6	0,5
	V3	1000	26,6	26,2	26,4	0,3
	V4	100	28,6	28,7	28,6	0,1
	V5	10	29,0	30,0	29,5	0,7
	V6	1	27,75	-	27,75	-

All six dilution steps of the reference RNA were able to be detected with the test assay in the FAM as well as in the Cy5 channel and were clearly distinguishable from the NTC, which indicates a LOD of 1 copy/RT-PCR

7.4 Accuracy

7.4.1 Methodology and samples:

The accuracy of the test assay was determined by performing following experiments:

- RT-PCR on 12 positive samples in duplicates
- RT-PCR on 48 negative samples in duplicates

The accuracy was tested by comparison of the sample results obtained by the test assay with those obtained with the validated inhouse assay.

7.4.2 Test and Results:

The results for the accuracy testing can be extracted from table 5 and show that the test assay recognized 23 out of 24 positive samples as “positive” and one (low-) positive sample as “negative” (sample number GB00016147).. All 48 negative samples were detected correctly as “negative” by the test assay. Taken together 71 out of 72 samples were identified correctly by the test assay, which corresponds to accuracy of ((23+48)x100)/72 = 98,7%.

7.5 Precision: Repeatability/Intermediate Precision

7.5.1 Methodology and samples:

The repeatability of the test assay was determined by performing following experiments:

- RT-PCR on following 10 positive samples in duplicates

The intermediate precision of the duplex assay was determined by performing following experiments:

- RT-PCR on following 10 positive samples in on two consecutive repetitions:

7.5.2 Test and Results:

Table 8: Results of the repeatability evaluation on 10 positive samples derived from repetition 1 of the intermediate precision test. The table displays the sample IDs, the Cp values of each duplicate, the mean Cp value of the duplicates and the standard deviation of the duplicate for each channel/assay.

A. Repetition 1

	FAM (N1-Assay)				Cy5 (E-gene Assay)			
Sample ID	**Cp1**	**Cp2**	**ØCp**	**SD**	**Cp1**	**Cp2**	**ØCp**	**SD**
GB00010736	29,03	29,53	29,28	0,35	27,68	27,88	27,78	0,14
GB00010749	28,46	28,41	28,44	0,04	27,06	26,98	27,02	0,06
GB00010774	24,63	24,08	24,36	0,39	23,59	22,98	23,29	0,43
GB00010779	25,95	26,52	26,24	0,40	24,35	24,79	24,57	0,31
GB00010819	28,37	27,88	28,13	0,35	27,19	26,77	26,98	0,30
GB00014809	21,19	21,10	21,15	0,06	20,58	20,49	20,54	0,06
GB00016290	26,54	26,47	26,51	0,05	24,59	24,35	24,47	0,17
GB00021151	20,49	20,29	20,39	0,14	18,34	18,22	18,28	0,08
GB00022879	28,25	28,46	28,36	0,15	27,73	27,81	27,77	0,06
GB00022881	31,10	31,59	31,35	0,35	28,89	28,95	28,92	0,04

Table 9: Results of the intermediate precision evaluation on 10 positive samples. The tables of repetitions 2 (B) displays the sample IDs, the Cp values of each duplicate, the mean Cp value of the duplicates and the standard deviation of the duplicates. The table 9C shows the combined data of all two repetitions and displays the sample numbers, the mean Cp values of each repetition, the mean Cp value of the repetitions and the standard deviation of the repetitions. Repetition 1 is displayed in table 8.

B. Repetition 2

	FAM (N1-Assay)				Cy5 (E-gene Assay)			
Sample ID	**Cp1**	**Cp2**	**ØCp**	**SD**	**Cp1**	**Cp2**	**ØCp**	**SD**
GB00010736	28,68	29,80	29,24	0,79	27,34	28,09	27,72	0,53
GB00010749	29,45	28,80	29,13	0,46	27,93	27,44	27,69	0,35
GB00010774	25,69	24,99	25,34	0,49	24,29	23,60	23,95	0,49
GB00010779	26,37	27,49	26,93	0,79	24,48	25,51	25,00	0,73
GB00010819	28,85	29,40	29,13	0,39	27,65	28,06	27,86	0,29
GB00014809	21,63	21,56	21,60	0,05	20,83	20,76	20,80	0,05
GB00016290	27,61	27,96	27,79	0,25	26,11	26,20	26,16	0,06
GB00021151	21,38	21,46	21,42	0,06	18,74	18,74	18,74	0,00
GB00022879	28,75	29,51	29,13	0,54	28,15	28,65	28,40	0,35
GB00022881	32,37	32,39	32,38	0,01	29,66	29,61	29,64	0,04

C. Combined (Repetition 1+2)

	FAM (N1-Assay)				Cy5 (E-gene Assay)			
Sample ID	**Rep 1**	**Rep 2**	**ØCp**	**SD**	**Cp1**	**Cp2**	**ØCp**	**SD**
GB00010774	29,28	29,24	29,26	0,03	27,78	27,72	27,75	0,05
GB00010802	28,44	29,13	28,78	0,49	27,02	27,69	27,35	0,47
GB00010819	24,36	25,34	24,85	0,70	23,29	23,95	23,62	0,47
GB00014809	26,24	26,93	26,58	0,49	24,57	25,00	24,78	0,30
GB00016289	28,13	29,13	28,63	0,71	26,98	27,86	27,42	0,62
GB00016290	21,15	21,60	21,37	0,32	20,54	20,80	20,67	0,18
GB00016471	26,51	27,79	27,15	0,91	24,47	26,16	25,31	1,19
GB00021151	20,39	21,42	20,91	0,73	18,28	18,74	18,51	0,33
GB00022879	28,36	29,13	28,74	0,55	27,77	28,40	28,09	0,45
GB00022881	31,35	32,38	31,86	0,73	28,92	29,64	29,28	0,51

The standard deviation of all 10 sample duplicates of repetition 1 (table 6) is below 0,5 Cps and therefore fulfills the acceptance criteria of repeatability.

The standard deviation of all sample repetitions is below 1,0 Cps (table 7C) and therefore fulfills the acceptance criteria of intermediate precision.

8 Deviations

N.A.

9 Discussion

In summary, the described experiments demonstrate that modifications of the validated inhouse assay have no significant impact on the RT-PCR performance of the SARS-CoV2 assay. The test assay has a specificity of 100%, a sensitivity of 96% and a accuracy of 98,7%. The acceptance criteria of precision was fulfilled and the LOD was evaluated with 1 copy/RT-PCR, which makes the tested assay conditions suitable to detect small residuals of RNA patient samples.

The SARS-CoV2 inhouse assay in combination with oligonucleotides and FAM, HEX and ATTO647N-Alabelled probes from Eurofins Genomics Europe Synthesis GmbH is hereby technically approved to be used for routine analysis.

10 Records

The analysis and documentation of the results are stored in a separate validation folder.

Location: 5.1.2h

Storage: 10 years, department APG

11 References and Related Documents

SOPs:

- SOP_APG_DNA-QuantiFluor_1.0
- SOP_APG_LightCycler480II_1.0
- SOP_APG_Methodenentwicklung_1.0
- SOP_APG_SARS-CoV-2_1.0

Analysis / Method Specification:

N.A.

References:

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- ICH Quality Guidelines Q2 (R1). Validation of Analytical Procedures: Text and Methodology. International Conference on Harmonization of Technical Requirements for the Registration of Drugs for Human Use. June 1995. CPMP/ICH/381/95
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- Mattocks CJ, Morris MA, Matthijs G, Swinnen, E, Corveleyen, A, Dequeker, E, et al. A standardized framework for the validation and verification of clinical molecular genetic tests. Eur J Hum Genet 2010;18:1276-88.
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- INSTAND e.V.; Extra INSTAND-Ringversuch (340) Virusgenom-Nachweis SARS-CoV-2 – April 2020; Zwischenauswertung vom 16.04.2020 (RvNr. 202003)

12 Attachments

Attachment 1: sample results derived from the test assay (repetition 1; RT-PCR plate PGX20_P0416). The table displays the sample ID, the Cp values of the dupllicates, mean Cp value of duplicates and standard deviation of duplicates. Results are shown for the N1- assay (FAM channel), the E-gene assay (Cy5 channel) and IPC specific signals (HEX channel).

	FAM (N1-Assay)				Cy5 (E-gene Assay)				HEX (IPC)			
Sample ID	**Cp1**	**Cp2**	**ØCp**	**SD**	**Cp1**	**Cp2**	**ØCp**	**SD**	**Cp1**	**Cp2**	**ØCp**	**SD**
GB00007638	-	-	-	-	-	-	-	-	27,54	-	27,5	-
GB00008816	35,62	39,15	37,39	-	-	-	-	-	27,56	26,87	-	-
GB00010626	-	-	-	-	-	-	-	-	28,27	28,3	28,3	0,02
GB00010634	-	-	-	-	-	-	-	-	27,98	29,50	-	-
GB00010654	-	-	-	-	-	-	-	-	20,91	28,45	-	-
GB00010736	29,03	29,53	29,28	0,35	27,68	27,88	27,78	0,14	-	27,93	27,93	-
GB00010741	-	-	-	-	-	-	-	-	27,95	28,55	28,25	-
GB00010744	-	-	-	-	-	-	-	-	27,74	28,16	27,95	0,30
GB00010745	-	-	-	-	-	-	-	-		27,7	27,70	-
GB00010748	-	-	-	-	-	-	-	-	28,76	28,47	-	-
GB00010749	28,46	28,41	28,44	0,04	27,06	26,98	27,02	0,06	27,78	27,38	27,58	-
GB00010768	-	-	-	-	-	-	-	-	27,19	28,76	27,98	1,11
GB00010774	24,63	24,08	24,36	0,39	23,59	22,98	23,29	0,43	24,94	24,31	-	-
GB00010775	-	-	-	-	-	-	-	-	28,46		28,46	-
GB00010778	-	-	-	-	-	-	-	-	28,53	28,83	28,68	-
GB00010779	25,95	26,52	26,24	0,40	24,35	24,79	24,57	0,31	24,56	25,79	-	-
GB00010802	23,46	22,85	23,16	0,43	22,10	21,62	21,86	0,34	23,56	22,58	-	-
GB00010816	-	-	-	-	15,26		-	-	28,5	28,45	28,48	0,04
GB00010819	28,37	27,88	28,13	0,35	27,19	26,77	26,98	0,30	-	27,26	27,26	-
GB00010821	-	-	-	-	-	-	-	-	28,29	28,58	-	-
GB00010822	-	-	-	-	-	-	-	-	28,35	28,53	28,44	-
GB00010823	-	-	-	-	-	-	-	-	27,59	28,19	27,89	0,42
GB00014809	21,19	21,10	21,15	0,06	20,58	20,49	20,54	0,06	18,81	-	-	-
GB00014812	-	-	-	-	-	-	-	-	-	26,45	-	-
GB00015711	40,28	43,75	-	-	-	-	-	-	-	-	-	-
GB00015772	-	-	-	-	-	-	-	-	27,64	26,98	-	-
GB00015774	36,74	36,86	36,80	0,08	-	-	-	-	27,54	29,79	28,67	-
GB00016014	-	-	-	-	-	-	-	-		19,25	-	-
GB00016075	-	-	-	-	-	-	-	-	27,71	27,10	-	-
GB00016076	-	-	-	-	-	-	-	-	27,42	-	-	-
GB00016083	-	-	-	-	-	-	-	-	26,51	-	-	-
GB00016147	-	15,78	-	-	-	-	-	-	26,08	26,07	-	-
GB00016289	30,08	30,60	30,34	0,37	26,48	26,05	26,27	0,30	26,95	-	-	-
GB00016290	26,54	26,47	26,51	0,05	24,59	24,35	24,47	0,17	-	25,16	-	-

GB00016471	23,05	22,98	23,02	0,05	20,72	20,73	20,73	0,01	20,42	21,65	-	-
GB00021150	-	38,14	38,14	-	-	-	-	-	28,1	27,88	27,99	-
GB00021151	20,49	20,29	20,39	0,14	18,34	18,22	18,28	0,08	17,98	17,05	-	-
GB00021155	-	-	-	-	-	-	-	-	26,23	-	-	-
GB00022865	-	-	-	-	-	-	-	-	28,63	-	-	-
GB00022875	-	-	-	-	-	-	-	-	28,54	-	28,54	-
GB00022879	28,25	28,46	28,36	0,15	27,73	27,81	27,77	0,06	-	27,15	27,15	-
GB00022881	31,10	31,59	31,35	0,35	28,89	28,95	28,92	0,04	28,91	-	28,91	-
GB00022882	-	-	-	-	-	-	-	-	27,93	28,23	-	-
GB00022886	-	-	-	-	-	-	-	-	27,45	28,81	28,13	-
GB00022904	-	-	-	-	-	-	-	-	28,69	27,81	28,25	0,62
GB00022911	-	-	-	-	-	-	-	-	28,49	28,02	-	-
GB00022912	-	-	-	-	-	-	-	-	-	28,65	-	-
GB00022917	-	-	-	-	-	-	-	-	28,54	28,33	28,44	-
GB00022936	-	-	-	-	-	-	-	-	28,23	28,23	28,23	0,00
GB00022945	-	-	-	-	-	-	-	-	28,16	27,72	27,94	-
GB00022947	17,33	-	-	-	-	-	-	-	28,19	29,19	28,69	-
GB00022951	-	16,88	-	-	-	-	-	-	-	28,34	28,34	-
GB00022955	-	-	-	-	-	-	-	-	28,68	28,26	28,47	-
GB00022969	-	-	-	-	-	-	-	-	28,32	-	-	-
GB00022971	-	-	-	-	-	-	-	-	28,54	28,75	28,65	0,15
GB00023070	31,76	32,79	32,28	0,73	29,50	29,73	29,62	0,16	27,68	28,96	28,32	-
GB00023079	-	-	-	-	-	-	-	-	29,03	28,16	28,60	-
GB00023156	-	-	-	-	-	-	-	-	28,44	28,59	28,52	-
GB00023181	-	-	-	-	20,74	30,53	-	-	29,34	29,3	29,32	0,03
GB00023901	38,26	38,09	38,18	-	19,28		-	-	-	-	-	-
GB00023913	26,06	25,66	25,86	0,28	24,03	23,48	23,76	0,39	25,24	24,57	-	-
GB00023914	-	-	-	-	-	-	-	-	27,72	24,57	-	-
GB00023919	-	-	-	-	-	-	-	-	16,50	-	-	-
GB00023975	34,59	37,76	36,18	2,24	-	-	-	-	26,29	26,93	-	-
GB00023977	-	-	-	-	-	-	-	-	23,68	26,18	-	-
GB00023980	-	-	-	-	-	-	-	-	26,08	17,61	-	-
GB00024138	34,77	34,48	34,63	0,21	-	-	-	-	27,44	26,7	-	-
GB00024278	-	-	-	-	-	-	-	-	-	23,74	-	-
GB00024279	24,79	24,57	24,68	0,16	23,70	23,45	23,58	0,18	24,28	-	-	-
GB00024282	-	-	-	-	-	-	-	-	16,81	25,25	-	-
GB00024285	-	-	-	-	-	-	-	-	27,45	-	-	-
GB00024289	32,47	33,21	32,84	0,52		28,27	-	-	30,24	27,59	-	-
GB00026034	-	-	-	-	-	-	-	-	25,28	-	-	-
NTC	-	-	-	-	-	-	-	-	27,88	28,14	-	-
NTC_EX	-	-	-	-	-	-	-	-	28,19	28,09	-	-

Attachment 2: sample results derived from the test assay (repetition 2; RT-PCR plate PGX20_P0418). The table displays the sample ID, the Cp values of the dupllicates, mean Cp value of duplicates and standard deviation of duplicates. Results are shown for the N1- assay (FAM channel), the E-gene assay (Cy5 channel) and IPC specific signals (HEX channel).

	FAM (N1-Assay)				Cy5 (E-gene Assay)				HEX (IPC)			
Sample name	**Cp1**	**Cp2**	**ØCp**	**SD**	**Cp1**	**Cp2**	**ØCp**	**SD**	**Cp1**	**Cp2**	**ØCp**	**SD**
GB00007638	-	29,93	29,93	#DIV/0!	-	-	-	-	27,41	28,19	27,80	0,55
GB00008816	34,65	34,60	34,63	0,04	30,56	30,17	30,37	0,28	27,74	27,61	27,68	0,09
GB00010626	-	-	-	-	-	-	-	-	28,80	28,75	28,78	0,04
GB00010634	-	-	-	-	34,29	-	34,29	-	28,69	28,28	28,49	0,29
GB00010654	-	-	-	-	-	32,80	32,80	-	28,68	28,66	28,67	0,01
GB00010736	28,68	29,80	29,24	0,79	27,34	28,09	27,72	0,53	28,42	28,34	28,38	0,06
GB00010741	33,19	-	33,19	-	-	-	-	-	28,63	28,85	28,74	0,16
GB00010744	-	27,31	27,31	-	-	-	-	-	28,43	27,90	28,17	0,37
GB00010745	-	32,54	32,54	-	-	-	-	-	28,76	28,53	28,65	0,16
GB00010748	-	35,67	35,67	-	-	-	-	-	28,51	28,65	28,58	0,10
GB00010749	29,45	28,80	29,13	0,46	27,93	27,44	27,69	0,35	27,85	28,57	28,21	0,51
GB00010768	-	-	-	-	-	35,85	35,85	-	28,27	29,12	28,70	0,60
GB00010774	25,69	24,99	25,34	0,49	24,29	23,60	23,95	0,49	28,09	24,60	26,35	2,47
GB00010775	33,44	-	33,44	-	32,84	29,20	31,02	2,57	28,59	28,77	28,68	0,13
GB00010778	-	-	-	-	-	-	-	-	29,13	28,73	28,93	0,28
GB00010779	26,37	27,49	26,93	0,79	24,48	25,51	25,00	0,73	27,14	26,93	27,04	0,15
GB00010802	25,23	24,98	25,11	0,18	23,26	23,02	23,14	0,17	25,94	24,94	25,44	0,71
GB00010816	-	-	-	-	-	-	-	-	28,54	28,18	28,36	0,25
GB00010819	28,85	29,40	29,13	0,39	27,65	28,06	27,86	0,29	28,49	28,16	28,33	0,23
GB00010821	-	-	-	-	-	-	-	-	28,67	28,65	28,66	0,01
GB00010822	35,30	6,68	20,99	20,24	-	-	-	-	28,97	28,53	28,75	0,31
GB00010823	-	29,55	29,55	-	31,94	30,33	31,14	1,14	28,55	29,10	28,83	0,39
GB00014809	21,63	21,56	21,60	0,05	20,83	20,76	20,80	0,05	-	23,87	23,87	-
GB00014812	-	-	-	-	-	-	-	-	27,48	28,12	27,80	0,45
GB00015711	38,70	37,97	38,34	0,52	-	8,74	8,74	-	28,77	27,47	28,12	0,92
GB00015772	30,39	-	30,39	-	-	-	-	-	28,28	27,57	27,93	0,50
GB00015774	36,81	37,92	37,37	0,78	-	-	-	-	28,36	28,56	28,46	0,14
GB00016014	-	31,02	31,02	-	34,15		34,15	-	28,20	28,25	28,23	0,04
GB00016075	-	-	-	-	-	-	-	-	28,09	27,68	27,89	0,29
GB00016076	-	-	-	-	-	-	-	-	28,26	26,98	27,62	0,91
GB00016083	30,63	-	30,63	-	33,97	28,86	31,42	3,61	28,14	27,29	27,72	0,60
GB00016147	38,34	37,26	37,80	0,76	27,56	29,74	28,65	1,54	28,61	28,12	28,37	0,35
GB00016289	31,37	31,35	31,36	0,01	28,49	28,55	28,52	0,04	27,88	28,44	28,16	0,40
GB00016290	27,61	27,96	27,79	0,25	26,11	26,20	26,16	0,06	27,44	27,61	27,53	0,12
GB00016471	24,19	23,89	24,04	0,21	21,32	21,25	21,29	0,05	23,56	24,46	24,01	0,64
GB00021150	5,00		5,00	-	-	-	-	-	27,98	28,18	28,08	0,14
GB00021151	21,38	21,46	21,42	0,06	18,74	18,74	18,74	0,00	-	20,28	20,28	-

GB00021155	-	40,39	40,39	-	-	29,55	29,55	-	28,78	28,06	28,42	0,51
GB00022865	-	14,65	14,65	-	32,01	-	32,01	-	28,53	28,49	28,51	0,03
GB00022875	-	-	-	-	-	-	-	-	28,78	28,20	28,49	0,41
GB00022879	28,75	29,51	29,13	0,54	28,15	28,65	28,40	0,35	27,80	28,63	28,22	0,59
GB00022881	32,37	32,39	32,38	0,01	29,66	29,61	29,64	0,04	28,13	28,86	28,50	0,52
GB00022882	31,03	-	31,03	-	-	28,33	28,33	-	28,91	28,25	28,58	0,47
GB00022886	33,60	-	33,60	-	45,00	-	45,00	-	28,46	28,18	28,32	0,20
GB00022904	-	-	-	-	-	-	-	-	28,67	28,58	28,63	0,06
GB00022911	-	-	-	-	31,23	29,63	30,43	1,13	28,47	28,84	28,66	0,26
GB00022912	28,42	7,69	18,06	14,66	-	-	-	-	28,73	28,38	28,56	0,25
GB00022917	-	-	-	-	-	-	-	-	28,64	28,90	28,77	0,18
GB00022936	-	28,62	28,62	-	-	-	-	-	28,32	28,52	28,42	0,14
GB00022945	-	-	-	-	32,89	32,86	32,88	0,02	28,52	28,72	28,62	0,14
GB00022947	-	-	-	-	-	-	-	-	29,15	28,61	28,88	0,38
GB00022951	37,37	37,97	37,67	0,42	31,19	31,18	31,19	0,01	28,80	28,37	28,59	0,30
GB00022955	29,22	-	29,22	-	30,89	30,95	30,92	0,04	28,59	28,64	28,62	0,04
GB00022969	-	30,48	30,48	-	-	-	-	-	28,22	28,27	28,25	0,04
GB00022971	-	-	-	-	-	-	-	-	-	28,92	28,92	-
GB00023070	33,89	33,85	33,87	0,03	30,18	30,33	30,26	0,11	29,17	28,83	29,00	0,24
GB00023079	-	-	-	-	-	-	-	-	28,62	28,27	28,45	0,25
GB00023156	31,35	-	31,35	-	-	-	-	-	28,61	28,46	28,54	0,11
GB00023181	30,14	37,23	33,69	5,01		45,00	45,00	-	28,65	28,50	28,58	0,11
GB00023901	45,00	37,76	41,38	5,12	-	-	-	-	27,96	27,81	27,89	0,11
GB00023913	27,24	27,27	27,26	0,02	25,30	25,35	25,33	0,04	27,16	27,08	27,12	0,06
GB00023914	-	-	-	-	-	-	-	-	27,91	27,79	27,85	0,08
GB00023919	-	-	-	-	-	-	-	-	27,86	28,01	27,94	0,11
GB00023975	34,71	35,40	35,06	0,49	30,17	30,16	30,17	0,01	28,36	27,99	28,18	0,26
GB00023977	-	-	-	-	-	-	-	-	28,77	27,81	28,29	0,68
GB00023980	-	28,55	28,55	-	-	30,01	30,01	-	28,09	28,32	28,21	0,16
GB00024138	33,67	34,27	33,97	0,42	30,34	30,74	30,54	0,28	28,41	28,27	28,34	0,10
GB00024278	-	31,68	31,68	-	-	-	-	-	28,46	27,94	28,20	0,37
GB00024279	25,61	25,61	25,61	0,00	24,68	24,64	24,66	0,03	27,08	26,12	26,60	0,68
GB00024282	31,02	-	31,02	-	-	-	-	-	28,34	28,63	28,49	0,21
GB00024285	-	-	-	-	-	-	-	-	28,51	28,15	28,33	0,25
GB00024289	32,92	32,82	32,87	0,07	30,21	30,12	30,17	0,06	28,52	-	28,52	-
GB00026034	-	-	-	-	-	-	-	-	27,66	27,92	27,79	0,18
NTC	-	-	-	-	-	14,50	14,50	-	27,84	27,98	27,91	0,10
NTC_EX	-	37,06	37,06	-	-	30,98	30,98	-	28,76	28,88	28,82	0,08

Attachment 3: sample results derived from the validated inhouse assay (repetition 1; RT-PCR plate PGX20_P0415). The table displays the sample ID, the Cp values of the dupllicates, mean Cp value of duplicates and standard deviation of duplicates. Results are shown for the N1- assay + E-gene Assay (FAM channel) and IPC specific signals (HEX channel).

	FAM (N1-Assay + E-gene Assay)				HEX (IPC)			
Sample ID	**Cp1**	**Cp2**	**ØCp**	**SD**	**Cp1**	**Cp2**	**ØCp**	**SD**
GB00007638	-	-	-	-	-	30,06	30,1	-
GB00008816	33,61	33,97	33,79	-	30,22	-	30,2	-
GB00010626	-	-	-	-	28,7	29,44	29,1	0,52
GB00010634	-	-	-	-	28,63	29,71	29,2	0,76
GB00010654	-	-	-	-	30,33	30,15	30,2	0,13
GB00010736	28,52	28,06	28,29	0,33	-	28,8	28,8	-
GB00010741	-	-	-	-	-	25,09	25,1	-
GB00010744	-	-	-	-	-	29,52	29,5	-
GB00010745	-	-	-	-	29,79	29,19	29,5	0,42
GB00010748	-	-	-	-		30,02	30,0	-
GB00010749	27,84	27,61	27,73	0,16	26,76	28,83	27,8	1,46
GB00010768			-	-	28,92	27,45	28,2	1,04
GB00010774	23,24	23,31	23,28	0,05	-	24,47	24,5	-
GB00010775	-	-	-	-	30,15	30,55	30,4	0,28
GB00010778	-	-	-	-	27,94	28,34	28,1	0,28
GB00010779	24,86	25	24,93	0,10	26,52	-	26,5	-
GB00010802	22,93	22,84	22,89	0,06	-	-	-	-
GB00010816	-	-	-	-	28,65	28,49	28,6	0,11
GB00010819	27,83	28,16	28,00	0,23	-	-	-	-
GB00010821	-	-	-	-	-	29,70	29,7	-
GB00010822	-	-	-	-	30,22	29,08	29,7	0,81
GB00010823	-	-	-	-	30,26	29,78	30,0	0,34
GB00014809	20,71	21,06	20,89	0,25		39,38	39,4	-
GB00014812	-	-	-	-	28,60		28,6	-
GB00015711	34,98	-	-	-	28,90	28,36	28,6	0,38
GB00015772	-	-	-	-	18,18		18,2	-
GB00015774	37,16	35,78	36,47	0,98	29,48	29,57	29,5	0,06
GB00016014	-	-	-	-	29,96	29,36	29,7	0,42
GB00016075	-	-	-	-	26,54	-	26,5	-
GB00016076	-	-	-	-	29,24	-	29,2	-
GB00016083	-	-	-	-	29,18	29,98	29,6	0,57
GB00016147	-	34,10	-	-	29,37	30,09	29,7	0,51
GB00016289	29,86	30,04	29,95	0,13	28,64	-	28,6	-
GB00016290	27,11	27,13	27,12	0,01	-	26,51	26,5	-
GB00016471	22,04	22,14	22,09	0,07	-	-	-	-
GB00021150	-	-	-	-	30,2	27,59	28,9	1,85
GB00021151	19,78	19,58	19,68	0,14	-	17,70	17,7	-

GB00021155	-	-	-	-	29,80	-	29,8	-
GB00022865	-	-	-	-	-	29,84	29,8	-
GB00022875	-	-	-	-	-	-	-	-
GB00022879	28,47	28,4	28,44	0,05	27,41	28,54	28,0	0,80
GB00022881	30,69	30,03	30,36	0,47	29,79	28,42	29,1	0,97
GB00022882	-	-	-	-	31,06	30,35	30,7	0,50
GB00022886	-	-	-	-	-	29,28	29,3	-
GB00022904	-	-	-	-	29,88	30,04	30,0	0,11
GB00022911	-	-	-	-	30,11	29,95	30,0	0,11
GB00022912	-	-	-	-	29,50	29,27	29,4	0,16
GB00022917	-	-	-	-	29,45	27,24	28,3	1,56
GB00022936	-	-	-	-	29,41	29,9	29,7	0,35
GB00022945	-	-	-	-	30,65	29,12	29,9	1,08
GB00022947	-	-	-	-	-	30,07	30,1	-
GB00022951	43,43	-	-	-	30,43	29,11	29,8	0,93
GB00022955	-	-	-	-	-	-	-	-
GB00022969	-	-	-	-	29,07	28,95	29,0	0,08
GB00022971	-	-	-	-	31,06	-	31,1	-
GB00023070	31,5	31,25	31,38	0,18	28,5	28,21	28,4	0,21
GB00023079	-	-	-	-	30,43	29,75	30,1	0,48
GB00023156	-	16,00	-	-	-	28,65	28,7	-
GB00023181	-		-	-	27,58	29,2	28,4	1,15
GB00023901	-	35,45	35,45	-	29,73	28,70	29,2	0,73
GB00023913	26,15	26,26	26,21	0,08	26,88	28,24	27,6	0,96
GB00023914	-	-	-	-	29,19	29,36	29,3	0,12
GB00023919	-	-	-	-	25,58	28,90	27,2	2,35
GB00023975	34,39	33,53	33,96	0,61	29,22	27,92	28,6	0,92
GB00023977	-	-	-	-	30,31	28,64	29,5	1,18
GB00023980	-	-	-	-	-	-	-	-
GB00024138	33,68	33,85	33,77	0,12	29,84	29,05	29,4	0,56
GB00024278	-	-	-	-	28,69	29,28	29,0	0,42
GB00024279	25,14	25,26	25,20	0,08	27,30	-	27,3	-
GB00024282	-	-	-	-	29,77	25,68	27,7	2,89
GB00024285	-	-	-	-	29,18	27,76	28,5	1,00
GB00024289	32,3	32,51	32,41	0,15	29,17	28,53	28,9	0,45
GB00026034	-	-	-	-	29,79	29,58	29,7	0,15
NTC	-	25,71	-	-	29,48	-	29,5	-
NTC_EX	-	-	-	-	-	31,05	31,1	-

Attachment 4: sample results derived from the validated inhouse assay (repetition 2; RT-PCR plate PGX20_P0417). The table displays the sample ID, the Cp values of the dupllicates, mean Cp value of duplicates and standard deviation of duplicates. Results are shown for the N1- assay + E-gene Assay (FAM channel) and IPC specific signals (HEX channel).

	FAM (N1-Assay + E-gene Assay)				HEX (IPC)			
Sample ID	**Cp1**	**Cp2**	**ØCp**	**SD**	**Cp1**	**Cp2**	**ØCp**	**SD**
GB00007638	-	33,84	-	-	29,47	29,52	29,50	0,04
GB00008816	33,89	33,58	33,74	-	29,73	29,16	29,45	0,40
GB00010626	-	33,66	-	-	30,03	30,1	30,07	0,05
GB00010634	-	-	-	-	30,16	-	30,16	-
GB00010654	-	-	-	-	28,89	28,74	28,82	0,11
GB00010736	29,55	29,06	29,31	0,35	29,10	28,89	29,00	0,15
GB00010741	-	-	-	-	29,88	30,32	30,10	0,31
GB00010744	-	-	-	-	28,95	28,69	28,82	0,18
GB00010745	-	31,00	-	-	29,77	29,45	29,61	0,23
GB00010748	-	-	-	-	30,09	30,58	30,34	0,35
GB00010749	29,09	28,75	28,92	0,24	28,83	28,8	28,82	0,02
GB00010768	-	-	-	-	29,82	29,99	29,91	0,12
GB00010774	24,34	24,87	24,61	0,37	24,79	25,29	25,04	0,35
GB00010775	-	32,24	-	-	29,9	29,72	29,81	0,13
GB00010778	-	-	-	-	29,81	30,13	29,97	0,23
GB00010779	26,19	26,68	26,44	0,35	26,96	27,40	27,18	0,31
GB00010802	23,85	24,24	24,05	0,28	24,06	25,02	24,54	0,68
GB00010816	-	-	-	-	29,9	29,57	29,74	0,23
GB00010819	28,49	29,11	28,80	0,44	28,73	28,88	28,81	0,11
GB00010821	32,03	-	-	-	30,05	29,96	30,01	0,06
GB00010822	-	-	-	-	29,55	-	29,55	-
GB00010823	-	-	-	-	29,9	29,89	29,90	0,01
GB00014809	21,35	21,3	21,33	0,04	21,34	21,58	21,46	0,17
GB00014812	-	-	-	-	28,84	28,71	28,78	0,09
GB00015711	36,27	37,19	-	-	29,33	29,12	29,23	0,15
GB00015772	-	-	-	-	28,81	29,18	29,00	0,26
GB00015774	35,5	-	35,50	-	29,51	28,7	29,11	0,57
GB00016014	-	-	-	-	29,07	29,55	29,31	0,34
GB00016075	-	-	-	-	29,18	29,50	29,34	0,23
GB00016076	-	-	-	-	28,79	29,08	28,94	0,21
GB00016083	-	-	-	-	28,62	29,18	28,90	0,40
GB00016147	-	35,17	-	-	28,73	29,15	28,94	0,30
GB00016289	30,07	30,25	30,16	0,13	29,06	29,00	29,03	0,04
GB00016290	27,34	27,31	27,33	0,02	27,63	27,63	27,63	0,00
GB00016471	22,62	22,52	22,57	0,07	22,78	22,91	22,85	0,09
GB00021150	37,14		-	-	29,84	-	29,84	-

GB00021151	19,85	19,82	19,84	0,02	20,27	18,99	19,63	0,91
GB00021155	-	-	-	-	29,14	29,41	29,28	0,19
GB00022865	-	-	-	-	29,96	29,77	29,87	0,13
GB00022875	-	-	-	-	30,00	29,46	29,73	0,38
GB00022879	28,9	29,02	28,96	0,08	28,94	28,75	28,85	0,13
GB00022881	30,94	31,85	31,40	0,64	29,6	29,75	29,68	0,11
GB00022882	-	-	-	-	29,85	29,62	29,74	0,16
GB00022886	-	-	-	-	29,93	29,24	29,59	0,49
GB00022904	-	-	-	-	-	29,28	29,28	-
GB00022911	-	-	-	-	29,92	29,67	29,80	0,18
GB00022912	-	-	-	-	29,77	29,71	29,74	0,04
GB00022917	-	-	-	-	30,28	29,65	29,97	0,45
GB00022936	-	-	-	-	30,02	30,15	30,09	0,09
GB00022945	-	-	-	-	30,14	29,99	30,07	0,11
GB00022947	-	-	-	-	28,92	29,98	29,45	0,75
GB00022951	36,29	-	-	-	29,85	30,18	30,02	0,23
GB00022955	-	-	-	-	29,61	30,04	29,83	0,30
GB00022969	-	-	-	-	29,84	29,64	29,74	0,14
GB00022971	-	-	-	-	29,39	29,70	29,55	0,22
GB00023070	32,69	32,86	32,78	0,12	30,16	29,91	30,04	0,18
GB00023079	-	-	-	-	29,80	29,78	29,79	0,01
GB00023156	-	-	-	-	30,00	28,93	29,47	0,76
GB00023181	-	-	-	-	29,9	29,82	29,86	0,06
GB00023901	-	37,04	37,04	-	29,18	29,61	29,40	0,30
GB00023913	26,39	26,28	26,34	0,08	27,15	27,20	27,18	0,04
GB00023914	-	-	-	-	29,00	28,91	28,96	0,06
GB00023919	-	-	-	-	29,07	29,48	29,28	0,29
GB00023975	34,14	33,86	34,00	0,20	29,22	29,17	29,20	0,04
GB00023977	-	28,76	-	-	29,63	29,10	29,37	0,37
GB00023980	-	-	-	-	29,53	29,44	29,49	0,06
GB00024138	34,49	34,62	34,56	0,09	29,66	29,92	29,79	0,18
GB00024278	-	-	-	-	29,82	29,32	29,57	0,35
GB00024279	25,54	25,47	25,51	0,05	25,26	26,44	25,85	0,83
GB00024282	-	-	-	-	29,36	29,33	29,35	0,02
GB00024285	-	-	-	-	29,32	28,95	29,14	0,26
GB00024289	32,97	32,84	32,91	0,09	29,49	29,42	29,46	0,05
GB00026034	-	-	-	-	-	29,57	29,57	-
NTC	-	-	-	-	29,26	29,10	29,18	0,11
NTC_EX	-	-	-	-	30,19	30,07	30,13	0,08