Validation of the detection of SARS-CoV-2 virus in nasopharyngeal swab specimens and samples collected by gargling in the primary and pooled specimens

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Content

1. Aim, specification of application, validation of method

The aim of the validation study was to validate the sensitivity of detection of the presence of SARS-CoV-2 virus in a pooled sample by two independent PCR detection methods.

2. Used chemicals and reagents

2.1. RNA isolation: Isolation was performed using a method based on paramagnetic beads CE IVD with the Viral Nucleic Acid Extraction Kit (Zybio Inc.; Cat. No. T200-96). The isolation was performed according to the manufacturer's recommended procedure **(Appendix No. 1).**

2.2. RT-PCR method: Two CE-IVD PCR detection kits were used to detect SARS-CoV-2 virus from isolated RNA:

2.2.1. Liferiver Novel Coronavirus (2019-nCoV) Real Time Multiplex RT-PCR Kit (Shangai ZJ Bio-Tech Co.) - hereinafter "Liferiver". Detection performed according to the manufacturer's instructions **(Appendix No. 2)**. The evaluation of the results was performed according to **Table 1**.

Table 1: Criteria for evaluating RT-PCR results of Liferiver Novel Coronavirus (2019-nCoV) Real Time Multiplex RT-PCR Kit

Result	**Criteria**
SARS-CoV-2 positive	If more than two FAM, HEX and Cal Red channels are detected and the amplification curves have a typical S shape and Ct <41, the sample is SARS-CoV-2 positive.
SARS-CoV-2 negative	Fluorescence channels FAM, HEX and Cal Red are not detected or Ct> 41 and Cy5 channel Ct <41, the sample is SARS-CoV-2 negative.
The result is unclear	If only one channel from FAM, HEX and Cal Red with Ct <is detected, it means that the result is in the grey zone and needs to be repeated. If the results are the same and show typical S-shaped curves, this is considered a positive result, otherwise a negative result.
Incorrect sample collection	Values of Ct> 41 or no value was measured in the fluorescence channels FAM, HEX and Cal Red and values of Ct≥ 41 or no value was measured in the channel Cy5, that is, that the result is invalid, and a new test is required.

2.2.2. SARS-CoV-2 Nucleic Acid Detection Kit (PCR-Fluorescent Probe Method; Zybio Inc.) - hereinafter "Zybio". Detection performed according to the manufacturer's instructions **(Appendix No. 3)**. The evaluation of the results was performed according to **Table 2**.

Table 2: Criteria for evaluating RT-PCR results of SARS-CoV-2 Nucleic Acid Detection Kit

Result	**Criteria**
SARS-CoV-2 positive	If more than two FAM, ROX and CY5 channels are detected and the amplification curves have a typical S shape and at least one Ct <40, this indicates that the sample is SARS-CoV-2 positive. FAM indicates the N gene, ROX indicates the RdRp gene and CY5 indicates the E gene.
SARS-CoV-2 negative	Fluorescence channels FAM, ROX and CY5 are not detected or Ct = 45 and VIC channel Ct <40, indicating that the sample is SARS-CoV-2 negative.
The result is unclear	If more than two channels from FAM, ROX and CY5 are detected but all Ct values are 40≤ Ct values <45, or only 1 channel with Ct <45 has been detected and VIC channel values have Ct values <40, this means that the result is in the grey zone and needs to be repeated. If the results are the same and show typical S-shaped curves, this is considered a positive result, otherwise a negative result.
Incorrect sample collection	Values of Ct =45 or no value was measured in the FAM, ROX and CY5 fluorescence channels and values of Ct ≥40 or no value was measured in the VIC channel, this means that the result is invalid and a new test is required.

3. Description of verification

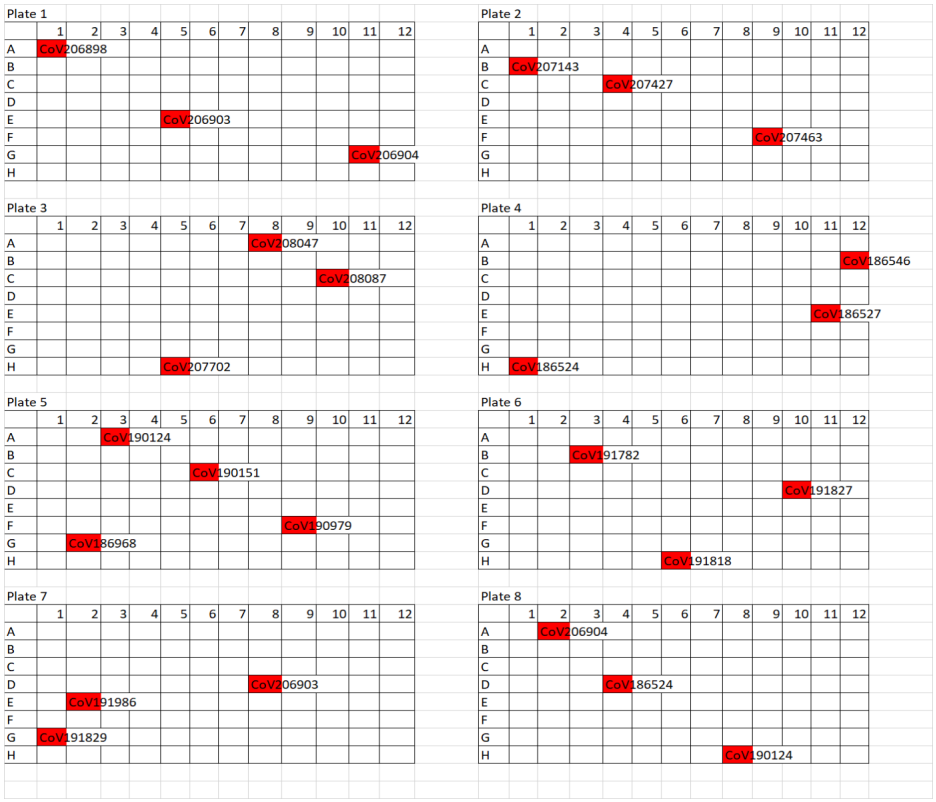
3.1. Verification with primary samples with Ct ≥ 35 in 3 independent pools (3 pooling axes).

For verification, 25 primary samples were used, in which Ct ≥35 was measured in at least one viral gene, which were examined using the Liferiver Novel Coronavirus detection kit (2019-nCoV) Real Time Multiplex RT-PCR Kit during routine diagnostic procedure **(see Table 3)**. These samples were supplemented with 743 SARS-CoV-2 negative samples. Biological materials were either from standard nasopharyngeal swabs or self-collected gargling samples (GARGtest, www.gargtest.com). The prevalence of SARS-COV-2 positivity in this part of the study was 3.26% (25/768). 150 µl of selected samples were distributed into eight 96-well plates according to **Scheme 1**. 150 µl of SARS-CoV-2 negative samples were pipetted into the other positions.

Table 3: Positively tested samples selected for the validation study (Ct and Cp values are also marked). Ct/Cp values indicate threshold values during routine diagnostic testing using the "Liferiver" kit.

Sample number	FAM530	HEX560	TR610	CY5-660	Result	Sample type
	Cp	Cp	Cp	Cp		
CoV206898	36.77	33.7	32.7	30.91	SARS Cov-2 positive	Nasopharyngeal swab
CoV206903	35.19	33.88	32.76	29.61	SARS Cov-2 positive	Nasopharyngeal swab
CoV206904	35.06	33.34	32.36	30.93	SARS Cov-2 positive	Nasopharyngeal swab
CoV207143	33.71	35.78	31.81	25.21	SARS Cov-2 positive	Nasopharyngeal swab
CoV207427	35.71	35.66	33.3	24.38	SARS Cov-2 positive	Nasopharyngeal swab
CoV207463	35.39	39.91	35.8	24.44	SARS Cov-2 positive	Nasopharyngeal swab
CoV207702	35.17	30.59	29.22	23.95	SARS Cov-2 positive	Nasopharyngeal swab
CoV208047	35.09	30.88	30	28.86	SARS Cov-2 positive	Nasopharyngeal swab
CoV208087	35.84	36.74	34.1	24.55	SARS Cov-2 positive	Nasopharyngeal swab
CoV186524	34.92	35.21	32.98	25.88	SARS Cov-2 positive	GARGtest
CoV186527	36.99	37.26	34.25	24.86	SARS Cov-2 positive	GARGtest
CoV186546	37.4	36.97	35.6	24.53	SARS Cov-2 positive	GARGtest
CoV186968	36.79	37.99	35.06	24.86	SARS Cov-2 positive	GARGtest
CoV190124	35.35	38.56	34.43	24.22	SARS Cov-2 positive	GARGtest
CoV190151	33.91	35.07	32.28	23.74	SARS Cov-2 positive	GARGtest
CoV190979	35.79	37.64	35.05	24.76	SARS Cov-2 positive	GARGtest
CoV191782	35.94	35.57	32.85	25.75	SARS Cov-2 positive	GARGtest
CoV191818	35	32.9	31.39	24.47	SARS Cov-2 positive	GARGtest
CoV191827	35.02	30.93	29.18	24.48	SARS Cov-2 positive	GARGtest
CoV191829	34.97	37.3	33.1	24.62	SARS Cov-2 positive	GARGtest
CoV191986	35.56	35.34	33.29	24.23	SARS Cov-2 positive	GARGtest

Scheme 1. Placement of SARS-CoV-2 positive samples in eight 96-well plates.



Eight prepared plates were pooled on EVO Freedom Tecan pipetting stations in three different axes - in the x-axis, the y-axis and the z-axis. By pooling in the “x-axis” (pooling by columns) 17 µl of sample from columns 1-12 from each 96-well plate were transferred into one column in the pooling plate. Thus, a pool of 1:12 was created, where all columns of the source plate 1 were in the first column of the pooled plate, all columns of the source plate 2 were in the second column of the pooled plate etc.

By pooling in the "y-axis", 25 µl of sample were transferred from rows A-H of each plate to individual rows of the pooled plate. Thus, a 1:8 pool was created, where all rows from the source plate 1 were in the first row of the pooled plate, all rows from the source plate 2 were in the second row of the pooled plate etc.

By pooling in the "z-axis", 25 µl of sample were transferred from positions 1A-12H of each plate to the same positions in the pooled plate. Thus, a 1:8 pool was created, where in the pooled 96-well plate were all the samples from positions 1A from all individual plates in position 1A, etc.

Each individual sample was contained in each of three independent pools. After pipetting the individual pooled plates, RNA was isolated from a 200 µl pooled sample using the Viral Nucleic Acid Extraction Kit and SARS-CoV-2 was detected by 2 independent PCR kits: Liferiver Novel Coronavirus (2019-nCoV) Real Time Multiplex RT-PCR Kit and SARS-CoV-2 Nucleic Acid Detection Kit (PCR-Fluorescent Probe Method; Zybio Inc.). The expected pooling results are shown in Scheme 2.

Scheme 2. Expected pooling results.

Pooled plate 3 (axis x) - columns													
	1	2	3	4	5	6	7	8	9	10	11	12	
A	positive	negative	positive	negative	positive	negative	negative	positive	no smpl.	no smpl.	no smpl.	no smpl.	
B	negative	positive	negative	positive	negative	positive	negative	negative	no smpl.	no smpl.	no smpl.	no smpl.	
C	negative	positive	positive	negative	positive	negative	negative	negative	no smpl.	no smpl.	no smpl.	no smpl.	
D	negative	negative	negative	negative	negative	positive	positive	positive	no smpl.	no smpl.	no smpl.	no smpl.	
E	positive	negative	negative	positive	negative	negative	positive	negative	no smpl.	no smpl.	no smpl.	no smpl.	
F	negative	positive	negative	negative	positive	negative	negative	negative	no smpl.	no smpl.	no smpl.	no smpl.	
G	positive	negative	negative	negative	positive	negative	positive	negative	no smpl.	no smpl.	no smpl.	no smpl.	
H	negative	negative	positive	positive	negative	positive	negative	positive	no smpl.	no smpl.	no smpl.	no smpl.	
	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6	Plate 7	Plate 8					
Pooled plate 2 (axis y) - rows													
	1	2	3	4	5	6	7	8	9	10	11	12	
A	positive	negative	negative	negative	positive	negative	negative	negative	negative	negative	positive	negative	Plate 1
B	positive	negative	negative	positive	negative	negative	negative	negative	positive	negative	negative	negative	Plate 2
C	negative	negative	negative	negative	positive	negative	negative	positive	negative	positive	negative	negative	Plate 3
D	positive	negative	negative	negative	negative	negative	negative	negative	negative	negative	positive	positive	Plate 4
E	negative	positive	positive	negative	negative	positive	negative	negative	positive	negative	negative	negative	Plate 5
F	negative	negative	positive	negative	negative	positive	negative	negative	negative	positive	negative	negative	Plate 6
G	positive	positive	negative	negative	negative	negative	negative	positive	negative	negative	negative	negative	Plate 7
H	negative	positive	negative	positive	negative	negative	negative	positive	negative	negative	negative	negative	Plate 8
Pooled plate 1 (axis Z) - positions													
	1	2	3	4	5	6	7	8	9	10	11	12	
A	positive	positive	positive	negative	negative	negative	negative	positive	negative	negative	negative	negative	
B	positive	negative	positive	negative	negative	negative	negative	negative	negative	negative	negative	positive	
C	negative	negative	negative	positive	negative	positive	negative	negative	negative	positive	negative	negative	
D	negative	negative	negative	positive	negative	negative	negative	positive	negative	positive	negative	negative	
E	negative	positive	negative	negative	positive	negative	negative	negative	negative	negative	positive	negative	
F	negative	negative	negative	negative	negative	negative	negative	negative	positive	negative	negative	negative	
G	positive	positive	negative	negative	negative	negative	negative	negative	negative	negative	positive	negative	
H	positive	negative	negative	negative	positive	positive	negative	positive	negative	negative	negative	negative	

3.2. Verification with primary samples with Ct 25≤FAM≥35 in pools 1: 8 and 1:12.

For further verification, 56 primary samples with Ct values of 25≤FAM≥35 were used, which were examined in routine diagnostics using the Liferiver Novel Coronavirus (2019-nCoV) Real Time Multiplex RT-PCR Kit (see Table 4).

Table 4: Positively tested samples selected for the validation study. Overview of positivity according to Cp and Ct values, respectively).

Sample number	FAM530	HEX560	TR610	CY5-660	Result	Sample type	Sample number	FAM530	HEX560	TR610	CY5-660	Result	Sample type
	Cp	Cp	Cp	Cp				Cp	Cp	Cp	Cp		
CoV190117	28.55	26.69	26.27	25.69	SARS Cov-2 positive	GARGtest	**CoV185388**	28.62	27.55	26.9	26.74	SARS Cov-2 positive	GARGtest
CoV190938	29.92	28.42	28.01	24.7	SARS Cov-2 positive	GARGtest	**CoV185394**	29.92	29.59	28.92	28.15	SARS Cov-2 positive	GARGtest
CoV190946	29.92	26.79	27.26	24.53	SARS Cov-2 positive	GARGtest	**CoV185491**	27.89	27.85	26.87	26.65	SARS Cov-2 positive	GARGtest
CoV190958	25.83	22.65	22.66	22.49	SARS Cov-2 positive	GARGtest	**CoV185501**	29.47	29.6	28.79	27.95	SARS Cov-2 positive	GARGtest
CoV190971	29.87	28.56	28.25	24.77	SARS Cov-2 positive	GARGtest	**CoV185503**	28.45	27.51	26.82	26.74	SARS Cov-2 positive	GARGtest
CoV190977	30.77	28.13	28.21	25.25	SARS Cov-2 positive	GARGtest	**CoV185505**	29.66	29.73	29	28.36	SARS Cov-2 positive	GARGtest
CoV190996	26.23	23.39	23.39	22.89	SARS Cov-2 positive	GARGtest	**CoV185510**	27.48	27.53	26.85	26.26	SARS Cov-2 positive	GARGtest
CoV190998	27.19	24.81	24.69	23.83	SARS Cov-2 positive	GARGtest	**CoV185514**	28.74	27.48	27.08	26.52	SARS Cov-2 positive	GARGtest
CoV190999	30.74	28.52	28.51	24.89	SARS Cov-2 positive	GARGtest	**CoV185516**	27.93	26.35	25.91	25.65	SARS Cov-2 positive	GARGtest
CoV191001	30.57	28.58	28.7	25.46	SARS Cov-2 positive	GARGtest	**CoV185530**	28.98	27.83	27.33	26.58	SARS Cov-2 positive	GARGtest
CoV191761	28.47	27.94	26.53	24.98	SARS Cov-2 positive	GARGtest	**CoV185531**	28.81	27.44	26.83	26.37	SARS Cov-2 positive	GARGtest
CoV191769	36.29	37.95	36.59	26.31	SARS Cov-2 positive	GARGtest	**CoV186136**	29.84	29.72	28.47	25.97	SARS Cov-2 positive	GARGtest
CoV191770	28.73	26.56	25.44	24.43	SARS Cov-2 positive	GARGtest	**CoV186148**	27.22	25.99	25.02	24.63	SARS Cov-2 positive	GARGtest
CoV191779	29.88	29.01	27.72	25.06	SARS Cov-2 positive	GARGtest	**CoV186159**	27.89	26.93	25.92	24.98	SARS Cov-2 positive	GARGtest
CoV191783	25.1	22.5	21.43	21.58	SARS Cov-2 positive	GARGtest	**CoV186162**	28.6	26.31	25.66	25.35	SARS Cov-2 positive	GARGtest
CoV191794	32.82	31.41	29.74	24.58	SARS Cov-2 positive	GARGtest	**CoV186164**	27.8	26.19	25.37	24.72	SARS Cov-2 positive	GARGtest
CoV191801	30.57	28.83	27.53	24.84	SARS Cov-2 positive	GARGtest	**CoV186501**	27.66	25.59	24.76	24.62	SARS Cov-2 positive	GARGtest
CoV191811	32.4	31.41	29.45	25.27	SARS Cov-2 positive	GARGtest	**CoV186521**	29.43	27.92	26.96	25.62	SARS Cov-2 positive	GARGtest
CoV191824	24.17	22.54	21.7	21.84	SARS Cov-2 positive	GARGtest	**CoV186533**	28.71	26.88	26.11	25.63	SARS Cov-2 positive	GARGtest
CoV191825	28.22	26.69	25.4	24.52	SARS Cov-2 positive	GARGtest	**CoV186545**	27.91	26.44	25.51	24.81	SARS Cov-2 positive	GARGtest
CoV191969	37.42	39.84	35.16	25.83	SARS Cov-2 positive	GARGtest	**CoV186971**	27.46	26.89	26.43	25.38	SARS Cov-2 positive	GARGtest
CoV191971	30.99	29.74	28.33	25.57	SARS Cov-2 positive	GARGtest	**CoV186990**	26.52	26.77	26.3	24.92	SARS Cov-2 positive	GARGtest
CoV191973	27.96	25.18	24.29	23.92	SARS Cov-2 positive	GARGtest	**CoV187016**	26.5	26.77	26.76	25.74	SARS Cov-2 positive	GARGtest
CoV191988	34.49	33.47	31.74	29.4	SARS Cov-2 positive	GARGtest	**CoV187023**	26.83	29.57	27.81	25.6	SARS Cov-2 positive	GARGtest
CoV191990	33.14	31.31	30.18	26.92	SARS Cov-2 positive	GARGtest	**CoV187771**	26.57	26.68	25.65	24.67	SARS Cov-2 positive	GARGtest
CoV185356	29.95	27.92	27.13	25.64	SARS Cov-2 positive	GARGtest	**CoV190134**	28.92	26.91	26.66	25.63	SARS Cov-2 positive	GARGtest
CoV185381	28.34	26.49 th most common	25.73	24.97	SARS Cov-2 positive	GARGtest	**CoV190138**	27.09	25.05	24.58	24.68	SARS Cov-2 positive	GARGtest
CoV185387	29.6	29.56	28.84	28.2	SARS Cov-2 positive	GARGtest	**CoV190159**	27.78	25.64	25.15	24.61	SARS Cov-2 positive	GARGtest

The selected 56 positive samples were pooled in a ratio of 1:8 (25 µl of positive sample + 7x25 µl of negative samples) and in a ratio of 1:12 (17 µl of positive sample + 11x17 µl of negative samples). Samples pooled this way were subjected to RNA isolation using the Viral Nucleic Acid Extraction Kit and SARS-CoV-2 detection using 2 independent PCR kits: Liferiver Novel Coronavirus (2019-nCoV) Real Time Multiplex RT-PCR Kit and SARS-CoV-2 Nucleic Acid Detection Kit (PCR-Fluorescent Probe Method; Zybio Inc.).

4. Results

4.1. Verification with primary samples with ct ≥ 35 in at least one gene in 3 independent pools (3 pooling axes).

4.1.1. Results of SARS-CoV_2 detection using Liferiver Novel Coronavirus (2019-nCoV) Real Time Multiplex RT-PCR Kit

Using the Liferiver kit, it was possible to detect 23 of 24 (95.8%) positive pools in the z-axis (pool 1:8). In 2/23 and 4/23, only one and two SARS-CoV-2 genes, respectively, were detectable.

In the y-axis (pool 1:8) we detected 21/25 (84%) positive pools. One and two SARS-CoV-2 genes, respectively, were detected in 1/21 and 2/21, respectively. In the x-axis (pool 1:12) we detected 19/25 (76%) positive pools. In 2/19 and 3/19, only one and two SARS-CoV-2 genes respectively, were detectable. The summary results are in **Scheme 3**, where positive samples are in red with detection of all 3 examined genes, yellow results with one or two positive genes and green false negative pools.

Scheme 3. Pooling results obtained with the Liferiver kit.

Pooled plate 3 (axis x) - columns

	1	2	3	4	5	6	7	8	9	10	11	12
A	3 genes	negative	3 genes	negative	negative	negative	negative	3 genes	-	-	-	-
B	negative	3 genes	negative	1 gene	negative	2 genes	negative	negative	-	-	-	-
C	negative	2 genes	2 genes	negative	negative	negative	negative	negative	-	-	-	-
D	negative	negative	negative	negative	negative	3 genes	3 genes	3 genes	-	-	-	-
E	3 genes	negative	negative	negative	negative	negative	1 gene	negative	-	-	-	-
F	negative	negative	negative	negative	negative	negative	negative	negative	-	-	-	-
G	3 genes	negative	negative	negative	3 genes	negative	3 genes	negative	-	-	-	-
H	negative	negative	3 genes	3 genes	negative	3 genes	negative	negative	-	-	-	-
	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6	Plate 7	Plate 8				

Pooled plate 2 (axis y) - rows

	1	2	3	4	5	6	7	8	9	10	11	12	
A	3 genes	negative	negative	negative	3 genes	negative	negative	negative	negative	negative	3 genes	negative	Plate 1
B	negative	negative	negative	3 genes	negative	negative	negative	negative	negative	negative	negative	negative	Plate 2
C	negative	negative	negative	negative	3 genes	negative	negative	3 genes	negative	negative	negative	negative	Plate 3
D	3 genes	negative	negative	negative	negative	negative	negative	negative	negative	negative	2 genes	2 genes	Plate 4
E	negative	negative	3 genes	negative	negative	3 genes	negative	negative	3 genes	negative	negative	negative	Plate 5
F	negative	negative	3 genes	negative	negative	3 genes	negative	negative	negative	3 genes	negative	negative	Plate 6
G	3 genes	1 gene	negative	negative	negative	negative	negative	3 genes	negative	negative	negative	negative	Plate 7
H	negative	3 genes	negative	3 genes	negative	negative	negative	3 genes	negative	negative	negative	negative	Plate 8

Pooled plate 1 (axis Z) - positions

	1	2	3	4	5	6	7	8	9	10	11	12
A	3 genes	3 genes	3 genes	negative	negative	negative	negative	3 genes	negative	negative	negative	negative
B	3 genes	negative	3 genes	negative	negative	negative	negative	negative	negative	negative	negative	1 gene
C	negative	negative	negative	3 genes	negative	3 genes	negative	negative	negative	negative	negative	negative
D	negative	negative	negative	3 genes	negative	negative	negative	3 genes	negative	1 gene	negative	negative
E	negative	3 genes	negative	negative	3 genes	negative	negative	negative	negative	negative	2 genes	negative
F	negative	negative	negative	negative	negative	negative	negative	negative	3 genes	negative	negative	negative
G	2 genes	3 genes	negative	negative	negative	negative	negative	negative	negative	negative	3 genes	negative
H	2 genes	negative	negative	negative	3 genes	3 genes	negative	2 genes	negative	negative	negative	negative

4.1.2. Results of SARS-CoV_2 detection using the SARS-CoV-2 Nucleic Acid Detection Kit (PCR-Fluorescent Probe Method; Zybio Inc.)

Using the SARS-CoV-2 Nucleic Acid Detection Kit (PCR-Fluorescent Probe Method; Zybio Inc.), 24 of 24 (100%) positive z-axis pools (pool 1:8) could be detected. In 3/24, only two SARS-CoV-2 virus genes were detectable. In the y-axis (pool 1:8) we detected 25/25 (100%) positive pools. Two SARS-CoV-2 genes were detected at 5/25. In the x-axis (pool 1:12) we detected 25/25 (100%) positive pools. At 2/25 and 6/25, only one and two SARS-CoV-2 genes, respectively, were detectable. The summary results are in **Scheme 4**, where clearly positive samples with detection of all 3 examined genes are highlighted in red, results with one or two positive genes are highlighted in yellow.

Scheme 4. Pooling results obtained with the Zybio kit.

Pooled plate 3 (axis x) - columns

	1	2	3	4	5	6	7	8	9	10	11	12
A	3 genes	negative	3 genes	negative	3 genes	negative	negative	3 genes	-	-	-	-
B	negative	3 genes	negative	2 genes	negative	2 genes	negative	negative	-	-	-	-
C	negative	2 genes	3 genes	negative	1 gene	negative	negative	negative	-	-	-	-
D	negative	negative	negative	negative	negative	3 genes	3 genes	3 genes	-	-	-	-
E	3 genes	negative	negative	3 genes	negative	negative	3 genes	negative	-	-	-	-
F	negative	1 gene	negative	negative	3 genes	negative	negative	negative	-	-	-	-
G	3 genes	negative	negative	negative	2 genes	negative	3 genes	negative	-	-	-	-
H	negative	negative	3 genes	3 genes	negative	2 genes	negative	2 genes	-	-	-	-
	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6	Plate 7	Plate 8				

Pooled plate 2 (axis y) - rows

	1	2	3	4	5	6	7	8	9	10	11	12	
A	3 genes	negative	negative	negative	3 genes	negative	negative	negative	negative	negative	3 genes	negative	Plate 1
B	3 genes	negative	negative	2 genes	negative	negative	negative	negative	2 genes	negative	negative	negative	Plate 2
C	negative	negative	negative	negative	3 genes	negative	negative	3 genes	negative	3 genes	negative	negative	Plate 3
D	3 genes	negative	negative	negative	negative	negative	negative	negative	negative	negative	3 genes	3 genes	Plate 4
E	negative	3 genes	2 genes	negative	negative	3 genes	negative	negative	2 genes	negative	negative	negative	Plate 5
F	negative	negative	3 genes	negative	negative	2 genes	negative	negative	negative	3 genes	negative	negative	Plate 6
G	3 genes	3 genes	negative	negative	negative	negative	negative	3 genes	negative	negative	negative	negative	Plate 7
H	negative	3 genes	negative	3 genes	negative	negative	negative	3 genes	negative	negative	negative	negative	Plate 8

Pooled plate 1 (axis Z) - positions

	1	2	3	4	5	6	7	8	9	10	11	12
A	3 genes	3 genes	3 genes	negative	negative	negative	negative	3 genes	negative	negative	negative	negative
B	3 genes	negative	3 genes	negative	negative	negative	negative	negative	negative	negative	negative	3 genes
C	negative	negative	negative	3 genes	negative	3 genes	negative	negative	negative	3 genes	negative	negative
D	negative	negative	negative	3 genes	negative	negative	negative	3 genes	negative	3 genes	negative	negative
E	negative	3 genes	negative	negative	3 genes	negative	negative	negative	negative	negative	2 genes	negative
F	negative	negative	negative	negative	negative	negative	negative	negative	3 genes	negative	negative	negative
G	3 genes	2 genes	negative	negative	negative	negative	negative	negative	negative	negative	3 genes	negative
H	2 genes	negative	negative	negative	3 genes	3 genes	negative	3 genes	negative	negative	negative	negative

Based on the positivity of the samples in the individual pools and their specific position, we were able to use the created macro to calculate the coordinates of a sample that is positive in a given pool with absolutely minimal need for additional examination of samples.

Examples:

1. Pool X - positivity 1A - positive sample is located in row A of the 1st plate, according to pool Y there are 3 possible positives, namely 1A, 5A, 11A. According to pool Z, it can be identified that this is position 1A. It is therefore sample CoV206898, which is located in the 2nd plate, in position 1A.
2. Pool X - positivity 7D - positive sample is located in row D of the 7th plate, according to pool Y there are 3 possible positives, namely 1D, 2D, 8D. According to pool Z, it can be identified that this is position 8D. It is therefore a sample of CoV206903, which is located in the 7th plate, in position 8D.

4.2. Verification with primary samples with Ct 25≤FAM≥35 in pools 1: 8 and 1:12.

4.2.1. Results of SARS-CoV_2 detection using Liferiver Novel Coronavirus (2019-nCoV) Real Time Multiplex RT-PCR Kit

Using the Liferiver kit, it was possible to detect all 1:8 pools, except for two pools (marked in green). Samples CoV191769 and CoV191969, which were not detectable in the pooled samples, had mean Ct values of 40.12 and 36.9. For samples pooled 1:12, 22/25 (88%) could be detected. These were the pools of samples CoV191769 and CoV191969 described above and sample CoV191779, whose average Ct value was 30.65.

Sample	Primary sample		Pool 1: 8		Pool 1:12							
number	FAM530	HEX560	TR610	CY5-IC	FAM530	HEX560	TR610	CY5-IC	FAM530	HEX560	TR610	CY5-IC
CoV185356	30.17	27.84	27.37	25.33	33.21	31.67	30.73	25.43	33.39	31.76	30.95	25.34
CoV185381	28.76	26.51	25.9	26.83	32.92	32.1	30.92	26.73	31.54	29.81	28.97	25.05
CoV185387	27.68	25.73	25.18	24.67	30.33	29.05	27.98	25.79	29.91	28.53	27.69	25.41
CoV185388	26.9	25.04	24.36	24.38	30.85	29.52	28.48	26.31	30.57	29.23	28.35	26.35
CoV185394	28.98	27.74	26.84	25.27	31.92	32.98	30.95	25.87	32.57	32.59	31.29	25.48
CoV185491	26.62	25.33	24.49	24.31	35.5	36.31	34.78	31.99	32.04	31.73	30.39	26.87
CoV185501	28.97	27.44	26.65	25.59	31.17	30.42	29.16	25.49	33.55	32.99	31.66	26.41
CoV185503	27.69	25.49	24.75	24.67	29.98	28.3	27.5	25.13	31.09	30.23	29.12	25.85
CoV185505	31.09	30.21	29.02	26.68	32.34	32.25	30.89	25.82	30.98	30.82	29.57	26.13
CoV185510	27.56	25.29	24.61	24.29	30.75	29.28	28.36	25.96	28.68	29.77	29.43	26.43
CoV185514	30.14	27.91	27.32	25.57	34.38	34.25	32.59	26.38	33.19	32.25	31.3	25.42
CoV185516	28.18	25.87	25.38	24.81	32.6	31.81	30.52	26.56	30.88	29.14	28.49	25.3
CoV185530	30.67	28.83	27.98	26.64	31.95	31.04	29.99	25.92	31.6	30.61	29.69	24.98
CoV185531	30.53	28.4	27.82	25.91	32.29	31.18	30.54	25.4	35.11	34.55	33.6	27.09
CoV186136	29.83	28.74	27.65	25.39	32.86	32.88	31.45	25.38	34.78	36.82	33.58	25.92
CoV186148	30.86	30.47	29.11	25.69	33.81	32.81	31.75	25.34	36.35	38.22	34.6	25.99
CoV186159	29.41	27.85	26.99	25.59	30.74	30.22	28.95	25.37	32.14	31.78	30.53	25.97
CoV186162	28.13	25.71	25.16	24.64	31.45	29.64	29.19	26.26	32.79	31	30.27	26.4
CoV186164	28.2	26.28	25.54	24.79	31.46	29.95	28.97	25.69	30.86	29.67	28.8	24.98
CoV186501	28.58	25.98	25.51	25.4	31.72	30.21	29.42	26.54	31.07	28.93	28.35	25.64
CoV186521	29.16	27.63	26.81	25.31	32.09	32.95	31.23	25.66	32.22	31.29	30.35	25.12
CoV186533	28.66	26.32	25.7	24.68	31.82	30.24	29.34	25.64	33.18	32.38	31.34	26.51
CoV186545	28.82	27.14	26.34	24.83	31.45	30.95	29.59	25.13	33.53	32.61	31.4	25.96
CoV186971	28.77	27.64	26.72	25.22	31.27	29.9	28.92	25.2	32.46	31.41	30.23	25.78
CoV186990	28.6	27	25.88	24.99	29.96	28.79	27.78	25.16	31.31	30.21	29.28	25.75
CoV187016	27.95	26.47	25.5	24.66	31.6	30.56	29.51	25.95	32.53	31.99	30.82	26.43
CoV187023	28.93	27.96	26.92	24.85	32.6	32.23	30.93	25.64	32.56	31.82	30.78	25.23
CoV187771	28.21	26.32	25.64	24.79	30.74	29.16	28.51	25.61	30.81	28.88	28.31	25.17
CoV190117	28.96	27.19	26.24	25.67	31.35	30.5	28.92	26	32.04	30.93	29.62	26.01
CoV190134	29.21	27.43	26.7	25.09	32.69	31.49	30.66	25.91	35.47	34.25	32.73	27.38
CoV190138	28.25	26.41	25.63	24.54	31.31	30.47	29.65	25.72	32.39	31.84	30.82	25.85
CoV190159	28.41	26.8	25.95	24.77	30.97	29.99	29.04	25.28	33.35	32.59	31.06	26.06
CoV190938	36.55	37.13	36.24	37.41	33.6	33.75	31.92	25.97	34.18	36.6	33.61	25.79
CoV190946	30.61	27.98	27.35	25.64	36.88	36.97	37.41	32.14	34.86	31.87	30.97	25.76
CoV190958	25.77	23.39	22.5	23.46	29.63	27.28	26.36	26.36	28.7	26.18	25.4	25.13
CoV190971	31.99	30.37	29.55	26.9	33.85	34.11	31.96	25.85	33.94	32.99	31.93	25.99
CoV190977	34.6	ND	ND	25.81	36.43	40.65	34.99	26.32	35.85	38.24	35.63	25.91
CoV190996	28.75	27.43	26.09	26.05	30.25	28.76	27.55	25.91	30.43	28.73	27.83	25.52
CoV190998	29.11	27.49	26.39	25.72	33.24	32.31	30.73	26.51	32.93	31.49	30.32	26.41
CoV190999	32.02	30.28	29.14	25.81	36.98	34.55	33.1	25.98	37.38	36.69	34.34	25.84
CoV191001	31.06	29.38	28.29	25.81	33.64	32.99	31.65	25.95	32.8	29.67	29.39	25.64
CoV191761	30.5	28.63	27.81	26.16	32.9	32.43	30.82	25.64	33.56	31.91	30.97	25.76
CoV191769	38.6	42.19	39.57	26.33	ND	ND	ND	26.29	ND	ND	ND	25.66
CoV191770	33.51	32.05	31.17	30.74	31.18	29.89 th most common	28.86	25.8	32.91	31.38	30.38	26.86
CoV191779	31.99	30.61	29.35	26.02	33.4	32.36	31.25	26.11	ND	ND	ND	30.91
CoV191783	26.18	24.97	23.87	24.11	28.99	26.99	25.97	25.42	29.06	26.84	26	25.75
CoV191794	36.85	35.79	34.4	25.99	37.48	39.53	38.5	26.1	ND	37.35	35.48	25.68
CoV191801	31.8	30.54	29.64	28.36	33.5	33.89	32.01	25.98	34.69	33.57	31.87	25.96
CoV191811	35.09	36.11	33.55	27.86	34.49	35.85	32.66	26.06	34.36	40.5	34.92	25.79
CoV191824	24.78	23.24	21.95	23.11	25.35	23.63	22.37	23.35	31.19	29.96	28.52	29.46
CoV191825	28.92	27.33	26.21	25.61	29.99	29.01	27.89	25.55	31.91	31.08	29.87	26.05
CoV191969	37.57	37.48	35.6	25.79	ND	ND	ND	25.59	ND	ND	40.62	25.94
CoV191971	32.88	31.29	29.96	26.14	34.62	37.5	34.83	26.17	36.56	36.34	33.78	25.95
CoV191973	27.21	24.62	23.77	24.5	29.78	27.35	26.46	25.63	30.07	27.73	26.87	25.76
CoV191988	30.98	29.82	28.7	26.21	33.98	34.02	31.97	25.97	34.53	35.72	33.54	26.49 th most common
CoV191990	31.97	30.87	29.35	25.78	36.99	36.76	33.76	25.95	34.94	36.66	34.85	25.97

ND - not detectable; IC - internal control

4.2.2. Results of SARS-CoV_2 detection using the SARS-CoV-2 Nucleic Acid Detection Kit (PCR-Fluorescent Probe Method; Zybio Inc.)

Using the Zybio kit, it was possible to detect all 1:8 and 1:12 pools except pools prepared from the CoV191969 sample (marked in green), whose average Ct value is 35.27.

Sample number	Primary sample				Pool 1: 8				Pool 1:12			
	FAM530	CY5 660	TR610	VIC560-IC	FAM530	CY5 660	TR610	VIC560-IC	FAM530	CY5 660	TR610	VIC560-IC
CoV185356	26.23	26.75	28.27	23.36	28.7	29.43	31.19	23.25	29.62	30.05	31.55	23.5
CoV185381	24.85	25.26	26.49 th most common	20.92	29.49 th most common	29.42	30.87	23.23	27.81	28.38	29.91	23.19
CoV185387	24.11	24.16	25.88	21.8	26.83	26.58	28.37	23.21	27.05	26.74	28.33	23.87
CoV185388	23.78	23.68	25.78	25.75	27.45	27.38	29.22	23.91	26.46	26.35	28.38	24.52
CoV185394	26.27	26.09	27.98	25.67	29	29.41	30.7	25.49	29.86	30.27	30.65	24.12
CoV185491	23.89	23.53	25.55	25.1	27.37	26.57	28.56	25.39	29.25	29.71	30.17	24.43
CoV185501	25.93	25.95	27.96	25.21	27.93	27.95	29.88	23.85	30.13	30.73	31.98	24.75
CoV185503	23.87	23.82	25.86	23.18	26.66	26.67	28.41	23.45	28.36	28.12	30.57	27.36
CoV185505	28.48	28.19	30.69	27.68	29.31	29.87	30.94	24.27	29.48	28.73	31.24	30.54
CoV185510	23.57	23.66	26.07	23.69	26.93	27.19	29.03	23.63	29.33	29.18	31.67	29.59
CoV185514	25.9	26.05	27.97	22.36	29.99	30.89	31.5	22.85	29.5	30.12	31.24	23.98
CoV185516	24.03	24.36	26.59	23.93	29.64	29.86	31	23.89	27.87	27.83	29.38	24.2
CoV185530	26.56	26.79	28.77	26.46	28.9	28.88	30.55	25.62	27.8	28.91	30.18	23.61
CoV185531	26.94	27.12	29.05	25.73	28.93	29.76	31.2	24.9	30.84	31.93	33.37	24.41
CoV186136	26.87	26.73	28.96	27.14	29.68	29.84	31.11	23.69	30.89	31.23	32.74	24.76
CoV186148	27.69	27.92	29.35	23.8	30.8	30.48	32.02	23.83	32.69	32.23	33.96	28.82
CoV186159	25.29	24.7	26.55	21.99	28.02	28.07	29	23.33	29.85	29.53	31.33	26.39
CoV186162	23.9	24.38	26.99	24.6	26.92	27.15	28.99	23.31	29.35	29.55	31.95	29.37
CoV186164	24.27	24.58	26.95	25.87	28.44	28.31	29.91	24.24	27.33	27.71	29.42	23.92
CoV186501	23.57	23.94	26.4	24.28	28.07	28.46	30.07	24.11	26.34	26.97	28.88	23.86
CoV186521	26.49 th most common	26.38	27.98	22.69	27.75	30.29	31.46	24.77	28.39	28.98	30.35	22.98
CoV186533	24.59	25.16	27.47	23.51	28.32	28.47	30.77	25.26	29.59	30.7	31.81	24.28
CoV186545	25.23	25.37	27.25	23.26	28.46	28.41	29.98	23.33	30.41	30.2	31.71	24.92
CoV186971	25.49	25.26	27.33	23.26	27.81	27.93	29.84	23.62	29.27	29.26	31.37	27.2
CoV186990	24.17	23.92	26.1	24.44	27.26	27.08	28.76	24.19	28.77	28.48	31.48	29.21
CoV187016	24.16	24.12	26.35	23.14	28.55	28.69	30.06	23.84	29.4	29.69	31.42	25.22
CoV187023	25.91	25.74	27.93	25.43	29.82	29.97	31.27	23.18	28.96	29.32	30.13	24.1
CoV187771	24.43	24.58	26.66	22.59	27.4	27.48	29.56	24.38	27.64	27.66	29.48	23.31
CoV190117	25.24	24.41	27.06	24.71	27.89	27.22	29.75	24.63	28.73	27.53	30.51	26.72
CoV190134	25.71	25.89	27.68	22.89	29.2	29.6	31.51	24.55	30.79	30.99	31.56	23.52
CoV190138	24.15	24.52	26.92	25.43	28.71	28.66	29.93	24.33	29.64	29.76	31.44	24.58
CoV190159	25.08	25.11	27.32	27.46	27.79	27.82	29.51	23.74	31.28	30.8	33.56	32.54
CoV190938	28.77	28.99	29.95	23.2	29.82	29.8	31.99	24.67	31.47	30.99	33.1	25.99
CoV190946	25.62	25.85	27.99	22.4	28.53	29.19	32.52	24.42	28.87	29.46	32.77	25.51
CoV190958	21.72	20.59	23.69	23.47	23.92	23.23	26.5	24.6	24.68	23.62	26.96	25.64
CoV190971	28.56	27.82	30.18	23.85	28.73	29.46	31.68	20.87	30.97	30.55	33.28	27.42
CoV190977	28.55	29.3	31.61	23.75	32.14	32.11	35.51	25.7	33.77	33.8	38.39	26.27
CoV190996	25.45	24.48	27.91	28.27	25.68	25.63	28.16	21.12	26.68	26.21	29.01	23.55
CoV190998	25.13	24.67	27.38	23.29	29.39	28.93	31.56	23.52	28.73	27.97	31.14	25.3
CoV190999	28.47	28.13	30.76	24.88	30.96	30.88	34.1	26.34	31.97	31.61	34.75	26.38
CoV191001	27.35	26.57	29.56	26.16	29.93	29.81	32.22	25.21	27.32	28.78	32.65	27.44
CoV191761	26.44	25.86	28.57	25.12	29.41	29.37	31.56	24.8	29.89 th most common	29.51	32.7	26.33
CoV191769	35.53	34.06	34.75	23.19	ND	35.04	ND	23.91	36.9	ND	ND	24.93
CoV191770	24.01	23.94	26.54	21.79	26.95	26.94	29.68	23.56	27.94	27.72	30.4	24.28
CoV191779	28.1	27.91	30.51	25.91	29.46	29.62	32.06	26.05	31.77	31.47	34.56	26.27
CoV191783	21.62	20.99	24.44	27.46	24.39	24.23	26.96	21.26	25.34	24.57	27.88	23.97
CoV191794	31.47	31.42	33.92	26.77	32.93	33.21	37.17	23.31	34.07	34.66	37.46	25.95
CoV191801	26.8	26.46	29.13	23.8	29.19	29.45	32.64	24.94	30.8	30.86	33.32	25.9
CoV191811	31.76	31.72	34.58	26.32	31.9	31.15	33.97	25.49	31.64	31.82	34.17	21.94
CoV191824	21.1	20	23.14	25.55	21.61	20.34	23.63	26.19	21.98	20.7	24.26	26.18
CoV191825	24.83	24.62	26.9	22.97	26.49 th most common	25.93	28.71	24.71	28.31	27.97	30.91	24.19
CoV191969	34.5	34.13	37.18	29.51	ND	ND	ND	26.28	ND	ND	ND	27.72
CoV191971	28.45	28.62	30.92	25.9	31.85	31.64	34.75	27.34	32.71	31.97	35.06	26.25
CoV191973	22.25	21.87	25.45	22.97	24.78	24.55	28.02	24.11	25.87	25.37	28.37	23.3
CoV191988	27.49	26.44	29.76	28.22	29.9	29.35	31.79	25.29	30.9	30.57	32.74	25.88
CoV191990	28.13	27.6	30.9	27.4	32.16	31.7	34.65	27.69	32.12	31.96	34.56	26.88

ND - not detectable; IC - internal control

5. Evaluation of results

The validation study tested the sensitivity of detecting the presence of SARS-CoV-2 virus in a total of 81 samples with Ct ≥ 25, which were pooled in a ratio of 1:8 and 1:12. The sensitivity of SARS-CoV-2 detection in pooled samples was tested by two independent PCR methods.

The results of the validation study unambiguously demonstrate that sufficient sensitivity can be achieved even with a pool of 1:12, provided that RNA isolation and a sufficiently sensitive PCR detection method are used. When comparing the two PCR detection methods, the sensitivity was clearly higher with the SARS-CoV-2 Nucleic Acid Detection Kit (PCR-Fluorescent Probe Method; Zybio Inc.). This is probably related to the larger volume of RNA sample (up to 50% of the reaction volume) that can be used in the PCR reaction in the Zybio kit compared to the Liferiver diagnostic kit. Due to the low prevalence of SARS-CoV-2 virus in the general population, in the context of preventive testing, it currently appears to be a sufficient pooling in the ratio of 1:12 with subsequent testing of positive pools. Testing should be performed on all pools, in which at least one of the three SARS-CoV-2 virus genes tested is amplified to achieve the highest possible sensitivity with the capture of positive samples with a lower viral load. Advantageously, high sensitivity PCR kits can be used to insert up to 50% of the volume of the RNA sample in water into the reaction volume and simultaneously detect up to three SARS-CoV-2 reference sequences.

In the case of a higher prevalence, it would be economically and operationally more advantageous to pool the samples in three axes, where the positive sample can in most cases be identified on the basis of the positivity in the individual pools.

This validation study also demonstrated advantages of non-invasive self-collection sampling using gargling technique, which enables collection of virus rich but less viscous materials than saliva into tubes without swabs, thus enabling high-throughput and fully automated testing.

6. Summary and conclusions

We declare that the method of pooling samples in a ratio of 1:8-12 followed by RNA extraction and detection of SARS-CoV-2 by sensitive PCR is sensitive and suitable for use in preventive testing for the presence of SARS-CoV-2 virus in nasopharyngeal swabs and self-collected materials sampled by GARGtest.

7. Authors of a validation study

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