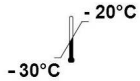
Test system for detection of SARS-CoV-2 (2019-nCoV) RNA in biologic substrates with Real-time RT-PCR

Instruction for use.

	Aimed for detection of SARS-CoV-2 genome fragments in biologic substrates for 50/100 tests	
	Unified format Is intended for use with thermal cyclers able to detect FAM/HEX fluorescence in 20 µl samples (CFX96, Rotor-Gene etc.)	(50/100 tests with controls)
	Recommended storage temperature	
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Content of the test system

Table 1. Components of test system

Label	Quantity (50 tests)	Quantity (100 tests)	Packing type	Description
PCR mixture	275 µl	550 µl	Screw Cap Tube	Mixture of reagents containing Taq-polymerase, dNTP, PCR-buffer and magnesium chloride
SARS-CoV-2 oligonucleotides	55 µl	110 µl	Screw Cap Tube	Mixture containing targeting primers and probes for SARS-CoV-2 and internal control
Reverse transcriptase	44 µl	88 µl	Screw Cap Tube	Mixture containing reverse transcriptase
SARS-CoV-2 positive control	100 µl	200 µl	PCR-tube	PCR-tube containing SARS-CoV-2 positive control
Water for PCR	1500 µl	1500 µl	Screw Cap Tube	Cleaned water for PCR
	1	1	Instruction	

Storage conditions, stability

Components of the kit (PCR mixture, SARS-CoV-2 oligonucleotides, reverse transcriptase, SARS-CoV-2 positive control, water for PCR) must be transported on dry ice, stored in dark place at –20…–30°C and must be placed there right after delivery. Stability of the kit is guaranteed within the whole storage period (when stored under mentioned above conditions). Reagents not included in the kit should be stored under the conditions recommended by their manufacturers.

Collection of samples

For detection of SARS-CoV-2 RNA it is possible to use the following biological substrates:

- nasopharyngeal and/or oropharyngeal swabs (in the presence of respiratory tract symptoms);
- sputum or bronchoalveolar lavage (in case of tracheal intubation);
- blood plasma;
- fecal matter (in the presence of symptoms of GIT lesion).

Reagents and equipment provided by the user

- thermal cycler for RT-PCR;
- computer with the pre-installed software of the thermal cycler for data analysis and logging;
- plastic consumables for thermal cycler;
- pipettes, sterile tips with aerosolic barrier for them;

- microcentrifuge suitable for 0.2 ml, 1.5 ml and 2.0 ml tubes and strips or 96-well plates;
- heating block/dry bath incubator suitable for 0.2ml, 1.5 ml and 2.0 ml tubes and 96-well plates;
- vortex;
- test-tubes of 0.2 ml, 1.5 ml and 2.0 ml;
- refrigerator from 2 to 8 °C with refrigerating chamber not higher than -18 °C;
- individual laboratory coat and disposable gloves;
- container with disinfecting solution.

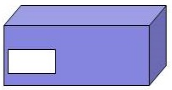
⚠ For work with NA it is necessary to use only disposable plastic consumables with special marking “RNase-free”, “DNase-free”.

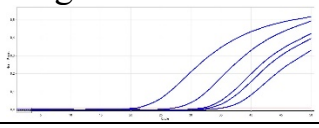
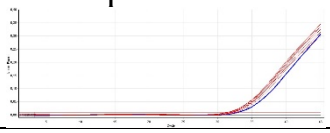
⚠ RNA is very sensitive to degradation by endogenous and exogenous RNAases that constantly present in the biological samples and environment. To achieve a satisfactory RNA output, contamination by ribonucleases should be dropped to a minimum. Avoid to work with bacterial cultures, cell cultures or other biological sources of RNAases in the same laboratory where RNA is isolated.

The principle of the kit for SARS-CoV-2 detection

The SARS-CoV-2 detection kit is a test system based on the RT-PCR principle. The kit is developed for detection of fragments of SARS-CoV-2 genome. Positive control must be amplified along with the analyzed samples.

Table 2. Brief review of the protocol.

1. Perform total RNA isolation using extraction kit		To perform the extraction in accordance with the manufacturer’s instructions of the extraction kit
2. Prepare and pour working solution of the reagent mixture into tubes, add samples		Prepare a reagent mixture at the rate of 12 µl in proportion to the number of analyzed samples (See also table 3). Add 12 µl of the working solution to all wells with test samples and controls. Add 8 µl of RNA samples to wells Add 8 µl of SARS-CoV-2 positive control to well with positive control, 8 µl of PCR water to the negative

	control well. Vortex and centrifuge for 5 seconds, 1,500g
3. Close the tubes, put them in the thermal cycler, set up and run RT-PCR	
4. Analysis of fluorescence signal	RNA concentration growth curves  Growth Curves of Internal Sample Controls 

Universal protocol for devices supporting FAM/HEX fluorescence detection

Important! Include at least 1 positive control and 1 negative control (Water for PCR) in each experiment. A negative control in which amplification occurred indicates contamination of samples with exogenous genetic material. In this case, it is necessary to repeat RT-PCR using freshly prepared reagents. Such samples must be examined at least twice in a row.

In order to maintain the full activity of the reagents, all operations for dosing and mixing reagents must be performed on ice or on a special cooled surface.

Table 3. Work stages. The list and volumes (µl) of reagents for the preparation of the working solution of the main reagent mixture.

1. Defrost reagents to room temperature. Centrifuge at 1500 g for 1 minute.

2. Briefly centrifuge tubes with PCR mixture with maximum speed before opening, so that the content guaranteed moves from the walls of the tubes to the bottom.

3. Preparation of the main reagent mixture.

- Vortex the prepared mixture for 3 seconds, then centrifuge for 5 seconds at full speed.
- It is recommended that 1-2 additional samples be added to the calculation to compensate for inaccuracies in dosing and pipetting losses.

Number of samples	1	2	3	4	5	6	7	8	9	10	11	12	13	14
SARS-CoV-2 oligonucleotides	1	2	3	4	5	6	7	8	9	10	11	12	13	14
PCR mixture	5	10	15	20	25	30	35	40	45	50	55	60	65	70
Reverse transcriptase	0,8	1,6	2,4	3,2	4,0	4,8	5,6	6,4	7,2	8,0	8,8	9,6	10,4	11,2
Water for PCR	5,2	10,4	15,6	20,8	26,0	31,2	36,4	41,6	46,8	52,0	57,2	62,4	67,6	72,8
Number of samples	15	16	17	18	19	20	21	22	23	24	25	26	27	28

SARS-CoV-2 oligonucleotides	15	16	17	18	19	20	21	22	23	24	25	26	27	28
PCR mixture	75	80	85	90	95	100	105	110	115	120	125	130	135	140
Reverse transcriptase	12,0	12,8	13,6	14,4	15,2	16,0	16,8	17,6	18,4	19,2	20,0	20,8	21,6	22,4
Water for PCR	78,0	83,2	88,4	93,6	98,8	104,0	109,2	114,4	119,6	124,8	130,0	135,2	140,4	145,6
Number of samples	29	30	31	32	33	34	35	36	37	38	39	40	41	42
SARS-CoV-2 oligonucleotides	29	30	31	32	33	34	35	36	37	38	39	40	41	42
PCR mixture	145	150	155	160	165	170	175	180	185	190	195	200	205	210
Reverse transcriptase	23,2	24,0	24,8	25,6	26,4	27,2	28,0	28,8	29,6	30,4	31,2	32,0	32,8	33,6
Water for PCR	150,8	156,0	161,2	166,4	171,6	176,8	182,0	187,2	192,4	197,6	202,8	208,0	213,2	218,4
Number of samples	43	44	45	46	47	48	49	50	51	52	53	54	55	56
SARS-CoV-2 oligonucleotides	43	44	45	46	47	48	49	50	51	52	53	54	55	56
PCR mixture	215	220	225	230	235	240	245	250	255	260	265	270	275	280
Reverse transcriptase	34,4	35,2	36,0	36,8	37,6	38,4	39,2	40,0	40,8	41,6	42,4	43,2	44,0	44,8
Water for PCR	223,6	228,8	234,0	239,2	244,4	249,6	254,8	260,0	265,2	270,4	275,6	280,8	286,0	291,2
Number of samples	57	58	59	60	61	62	63	64	65	66	67	68	69	70
SARS-CoV-2 oligonucleotides	57	58	59	60	61	62	63	64	65	66	67	68	69	70
PCR mixture	285	290	295	300	305	310	315	320	325	330	335	340	345	350
Reverse transcriptase	45,6	46,4	47,2	48,0	48,8	49,6	50,4	51,2	52,0	52,8	53,6	54,4	55,2	56,0
Water for PCR	296,4	301,6	306,8	312,0	317,2	322,4	327,6	332,8	338,0	343,2	348,4	353,6	358,8	364,0
Number of samples	71	72	73	74	75	76	77	78	79	80	81	82	83	84
SARS-CoV-2 oligonucleotides	71	72	73	74	75	76	77	78	79	80	81	82	83	84
PCR mixture	355	360	365	370	375	380	385	390	395	400	405	410	415	420
Reverse transcriptase	56,8	57,6	58,4	59,2	60,0	60,8	61,6	62,4	63,2	64,0	64,8	65,6	66,4	67,2
Water for PCR	369,2	374,4	379,6	384,8	390,0	395,2	400,4	405,6	410,8	416,0	421,2	426,4	431,6	436,8
Number of samples	85	86	87	88	89	90	91	92	93	94	95	96	97	98
SARS-CoV-2 oligonucleotides	85	86	87	88	89	90	91	92	93	94	95	96	97	98
PCR mixture	425	430	435	440	445	450	455	460	465	470	475	480	485	490
Reverse transcriptase	68,0	68,8	69,6	70,4	71,2	72,0	72,8	73,6	74,4	75,2	76,0	76,8	77,6	78,4
Water for PCR	442,0	447,2	452,4	457,6	462,8	468,0	473,2	478,4	483,6	488,8	494,0	499,2	504,4	509,6
Number of samples	99	100	101	102	103	104	105	106	107	108	109	110		
SARS-CoV-2 oligonucleotides	99	100	101	102	103	104	105	106	107	108	109	110		
PCR mixture	495	500	505	510	515	520	525	530	535	540	545	550		
Reverse transcriptase	79,2	80,0	80,8	81,6	82,4	83,2	84,0	84,8	85,6	86,4	87,2	88,0		
Water for PCR	514,8	520,0	525,2	530,4	535,6	540,8	546,0	551,2	556,4	561,6	566,8	572,0		

4. Prepare enough tubes to amplify the test samples, positive and negative controls and place them in a refrigerated stand.

5. Add 12 µl of the working solution of the main reagent mixture to all tubes with test samples and controls.

6. Transfer 8 µl of water for PCR to the negative control tube containing no samples and 8 µl of positive control to the positive control tube.

7. Add 8 µl of RNA samples to the appropriate tubes.

8. Close the tubes with caps, strips, or ultratransparent film.

9. Shake (using shaker) and then displace the liquid to the bottom of the tubes using centrifuge for 10 seconds.
10. Carefully place the prepared tubes in the thermal cycler, close the lid.
11. Run the PCR protocol setup following the manufacturer's recommendations, taking into account the differences in the settings of your instrument. Create a new protocol or start the existing one. Target the moment of reading the data from the plate at the phase **annealing/elongation** at 59 °C. Indicate that the FAM (SARS-CoV-2) and HEX (endogenous internal control) channels should be used in this experiment. Indicate the used wells on the plate diagram. Type the name of the corresponding sample in each well.

Table 4. Amplification conditions for SARS-CoV-2 RNA detection.

Step	Temperature	Time	Number of repeats
Reverse transcription	**50 °C**	**20 minutes**	**1**
Activation of polymerase, inactivation of reverse transcriptase	**95 °C**	**3 minutes**	**1**
Denaturation	**95 °C**	**15 seconds**	**5**
Annealing	**59 °C**	**20 seconds**	
Elongation	**72 °C**	**10 seconds**	
Denaturation	**95 °C**	**15 seconds**	**40**
Annealing and measuring of fluorescence	**59 °C**	**20 seconds**	
Elongation	**72 °C**	**10 seconds**	

Total time necessary for finishing of RT-PCR in accordance with this protocol is approximately 1 hour 30 minutes.

Data analysis

Each cycle of RNA amplification leads to the generation of a fluorescent signal measured in FAM channel for target leads to the formation of a sigmoid melting curve. Data analysis should be performed in accordance with the recommendations of the equipment manufacturer using compatible software.

1. With a FAM Ct value less than 40, and a HEX Ct value less than 40, the sample contains fragments of the SARS-COV-2 genome (positive).
2. In the absence of (N/A) of Ct value on the FAM channel and Ct value on the HEX channel less than 40, the sample does not contain fragments of the SARS-COV-2 genome (negative).
3. With a FAM Ct value less than or equal to 20 and no HEX Ct value (N/A), the sample contains fragments of the SARS-COV-2 genome (positive).

4. If the FAM Ct value is more than 20, and the HEX Ct value is absent (N/A), it is necessary to repeat the RT-PCR of the sample, including two ten-fold dilutions. If in one of the samples sigmoid curves are found on the FAM and HEX channels, the sample contains fragments of the SARS-COV-2 genome (positive).
5. If there is no Ct value on the FAM (N/A) channel and no Ct value on the HEX (N/A) channel, the result is considered invalid. It is necessary to repeat the study of the sample, including the extraction step.

⚠ In case of getting ambiguous positive results there is possible instruments and/or working place contamination - decontamination measures in the laboratory should be carried out.

⚠ The set detection threshold level can significantly affect Ct values. Set up threshold levels in accordance with recommendations of the manufacturer.

Solution of the problems

Table 5. Possible Causes of Errors and their Solutions

Problem	Possible cause	Solution
Samples Issues		
Inadequate purity of extracted RNA	Contamination of RNA/DNA samples with protein salts, carbohydrates and other organic matters that inhibit PCR	Avoid phenolic and/or other extraction methods, use only the included nucleic acid extraction kit.
Pipetting Issues		
A fluorescent signal was obtained from samples containing no DNA and/or from reagent contamination controls	Contamination of negative probes with amplicons	Repeat extraction and/or PCR with new reagents; decontaminate the instruments and working place.
The total volume of the reaction mixture differs from 20 µl	Pipetting errors, for example, skipping or refilling cells	Use multichannel pipettes, automated pipetting or develop attention and concentration.
Amplification Issues		
Unusually high C_T standard values and/or high RNA concentration in test	Invalid amplification protocol	Check the equipment settings,follow the instructions from the device operation guide

samples	Nonobservance of storage conditions and/or expiry dates of the reagents	Check storage conditions and expiry date
	NA breakdown	Use nuclease-free supplies and reagents; after synthesis, immediately place RNA samples on ice
Non-sigmoid shape of melting curves of concentration standards and tested samples	Frequent defrosting or improper storage of dissoluted reagents mixture	Read instruction, check storage conditions, prepare fresh reagents mixture.
	Storage conditions do not correspond to recommended, the set is expired	Check storage conditions and expiry date.
Absence of fluorescent signal	Measuring of fluorescent signal is off, camera is improperly installed	Check the equipment settings
	Incorrect channel of fluorescent signal recording has been chosen	To identify RNA of the pathogen choose channel FAM. To define internal control choose channel HEX
	Invalid amplification protocol	Check the equipment settings
	Nonobservance of storage conditions and/or expiry dates of the kit	Check storage conditions and expiry date.
Different types of RNA amplification in test samples, unparalleled growth of curves in the exponential phase of the reaction	Excess PCR inhibitors in the sample	Use the recommended extraction kit, exactly follow the manufacturer's instructions. Dilution of RNA before analysis can reduce the amount of inhibitors in the sample
	Improperly collected material (e.g. heparinized blood)	Use correctly collected samples.

A low level of fluorescent signal is registered in the process of identifying RNA amplification	Contamination of optical lenses	See “care” section for instructions on using the appropriate thermal cycler; if the design allows, wipe the lens once a month using absolute ethanol and cotton swabs.
	Contamination of cooling system and/or optical sensor matrix	See the “care” section for instructions on using the appropriate thermal cycler; you can also fill each well of the sensor with isopropanol, incubate for 10 minutes at 50 °C, remove isopropanol and rinse with bidistilled water.