DirectFast™ SARS-CoV-2 multiplex assay
(Cat. No. DFSCV100-D)

Instructions for Use

IVD

For *in vitro* diagnostic use only
For professional use only.

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1. Intended Use

'*DirectFast™* SARS-CoV-2 multiplex assay' is an *in vitro* diagnostic test for the qualitative detection of SARS-CoV-2 RNA by real-time RT-PCR (reverse-transcription polymerase chain reaction). This assay targets specifically the ORF8, N, and S genes of SARS-CoV-2 in the extracted nucleic acids from upper respiratory samples (nasopharyngeal swab, oropharyngeal swab) of individuals suspected of COVID-19. The product is optimized for Applied Biosystems™ 7500(Fast) PCR Instrument system and Bio-Rad CFX96™ Real-time PCR detection system. '*DirectFast™* SARS-CoV-2 multiplex assay' is intended for use by professionals of real-time RT-PCR and *in vitro* diagnostics.

2. General Principles

'*DirectFast™* SARS-CoV-2 multiplex assay' is a multiplex real-time reverse transcription polymerase chain reaction test using dual-labeled probes. For the detection of SARS-CoV-2 RNA, primers and probes are designed to bind specifically to ORF 8, N, and S genes. Detection of the multiple targets lowers the risk of false-negative results induced by mutations of viral RNA. To prevent the carryover contamination of amplified products, this assay uses dUTP and heat-labile uracil-N-glycosylase (UNG) enzymes.

2.1 Applicable real-time PCR instrument

Instrument
Bio Rad CFX96™ Real-time PCR detection system
Applied Biosystems™ 7500(Fast) PCR Instrument system

2.2 Target genes and fluorescence chemistry of probes

Target gene	S	ORF8	N	RNase P(IC)
Fluorescence chemistry of probe	FAM	JOE	Texas Red	Cy5

2.3 Template (Nucleic acid preparation)

For the reaction templates, the nucleic acids purified using commercially available viral RNA preparation kits can be used. The crude extracts of swab samples which collected in UTM (universal transport medium, or VTM) by heating (for 5 minutes at 92~95°C) or using the '10x nucleic acid extraction solution' (NAExDB, NanoHelix) are also applicable as a template to this assay.

3. Warnings & Precautions

- For in vitro diagnosis use only.
- For professional use only.
- All patient specimens and positive controls should be considered infectious and biohazardous and handled accordingly with safe laboratory procedures.
- Use personal protective equipment (PPE) consistent with current guidelines for the handling of potentially infectious samples.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in areas where reagents and human specimens are handled.
- Do not use products beyond the expiration date.
- Store the product below -20 °C.
- Do not mix with other product numbers.
- Always wear laboratory gloves, lab coats, and goggles when handling the product to protect it from reagents or samples.
- Use RNase-free, DNase-free tips with the aerosol barrier to prevent contamination.
- All reagents are taken out immediately before use, thawed, and used after centrifugation for several seconds.
- After using the reagents, immediately store below -20 ℃, and limit freezing and thawing to five (5) times.
- When you handle the tube or cap, wear vinyl gloves or rubber gloves without powder.
- If the tube is not well closed with a cap, the contents may evaporate and abnormal results may occur.
- Check if the real time PCR conditions and fluorescence dye selection described in the inspection method are correctly set before proceeding.
- PCR is a very sensitive method, so be careful of carryover contamination.
- Dispose of unused reagents, waste, and samples according to regulations.
- If reagent gets into your eyes, immediately rinse with water and follow doctor's instructions.
- If reagent comes into contact with skin, immediately rinse with water.
- When handling samples that may cause infection, treat them safely according to CLSI Guideline M29-A.
- Real time PCR instrument is managed periodically by the manufacturer's instructions.

4. Product Components

No.	Component	Cap	Description	Volume (per 100 Tests)
1	Reaction Mix (SCV2)	RM	Buffer mix (5x conc.)	400 µL x 1 tube
2	Enzyme Mix (SCV2)	EM	Mixture of enzymes	200 µL x 1 tube
3	Oligo Mix (SCV2)	OM	Mixture of primers and probes	400 µL x 1 tube
4	RNase free Water	RNase free Water	Distilled, sterile, RNase-free/DNase-free water	1,000 µL x 1 tube
5	PC (SCV2)	PC	Positive Control DNA	50 µL x 1 tube

5. Storage & Stability

- Store the products below -20 °C.
- The shelf life is 12 months from the date of manufacture.
- Freezings and thawings are limited to five (5) times.
- Please use the reagents within 30 days after opening.

6. Additional required Instrument and Materials

Instrument	Bio-Rad CFX96™ Real-time PCR detection system (software version 1.6)
	Applied Biosystems™ 7500(Fast) PCR Instrument system (software version 2.0.6)
Materials	Micropipettes, adjustable Aerosol barrier, RNase- and DNase-free tips PCR tube (8-tube/cap strip, 96 well plate) • Low-Profile PCR Tubes 8-Tube Strip, white (Cat. no. TLS0851, Bio-Rad) • Optical Flat 8-Cap Strips (Cat. no. TCS0803, Bio-Rad) • MicroAmp Optical 8-Tube Strip(0.2 mL) (REF 4316567, ABI) • MicroAmp Fast Reaction Tube(8 Tubes/Strip) (REF 4358293, ABI) • MicroAmp Optical 8-Cap Strip (REF 4323032, ABI) • MicroAmp Fast Optical 96-Well Reaction Plate (REF 4346907, ABI) • MicroAmp Optical 96-Well Reaction Plate (REF N8010560, ABI)

	Microtubes 1.5ml or 2ml Latex Gloves Vortex mixer Micro Centrifuge

7. Sampling and Handling

- All specimens should be considered infectious and biohazardous and handled accordingly with safe laboratory procedures.
- Upper respiratory samples (Nasopharyngeal swab, oropharyngeal swab) are the specimens for this test.
- For specimen collection and handling, refer to CDC Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens from Persons for Coronavirus Disease 2019 (COVID-19). (https://www.cdc.gov/coronavirus/2019-nCoV/lab/guidelines-clinical-specimens.html)
- Use UTM (universal transport medium, or VTM) for swab collection and storage for the use of '10x Nucleic acid extraction solution' (NanoHelix, Republic of Korea) for template preparations.
- If specimens will be examined within 48 hours after collection, keep specimen at 4~8 °C. If a delay in testing or shipping is expected, store specimens at -70 °C or below.
- Avoid freezing and thawing specimens. Viability of some pathogens from specimens that were frozen and then thawed is greatly diminished and may result in false-negative test results.

8. Nucleic acid Extraction

8.1 Nucleic acid extraction method

Template nucleic acids can be prepared by one of these procedures.

(a) The commercially available nucleic acid extraction procedures that have been shown to generate highly purified RNA, including PureHelix™ Viral DNA/RNA Kit V2 (Cat. VNK200D, NanoHelix) and QIAamp® DSP viral RNA mini Kit (Cat.61904, QIAGEN). Users should follow the manufacturer's recommendation for use.

(b) A fast and straightforward nucleic acid preparation procedure using the '10x Nucleic

acid extraction solution (cat. NAExDB, NanoHelix)'. Users should follow this procedure:

① Take 90 µL of specimen collected in UTM (universal transport medium or VTM) and place it in a 1.5 ml tube.

② Add 10 ul of 10X Nucleic acid extraction solution.

③ Mix gently by tapping or vortexing and spin down for a few seconds.

④ Use 3ul as a nucleic acid sample (PCR template) per reaction.

(C) Heating procedure:

① Mix well the specimen collected in UTM (universal transport medium or VTM) by vortexing or pipetting.

② Take 50 µL of the specimen and place it in a 1.5 ml tube. Close the tube cap tightly.

③ Transfer the tube on a dry bath (heat-block) that pre-heated to 92~96°C.

④ After 5 minutes of heating, take out the tube and make cool at room temperature.

⑤ Mix gently by tapping or vortexing, and centrifuge for 2 minutes.

⑥ Use 3ul of the supernatant as a nucleic acid sample (PCR template) per reaction.

8.2 Quality control of nucleic acid extraction

- A negative extraction control (NEC) is a previously characterized negative patient sample. It serves both as a negative extraction control to monitor for any cross-contamination that occurs during the extraction process, as well as an extraction control to validate extraction reagents and successful nucleic acid extraction.
- An internal control targeting human RNaseP mRNA is needed to monitor if any potential PCR inhibitor exists in the specimen and is used through the entire sample processing procedure. Oligo Mix (SCV2) in '*DirectFast™* SARS-CoV-2 multiplex assay' contains primers and probes for targeting human RNaseP mRNA as an internal control.

9. Protocol

9.1 Reagent Preparation

- All reagents, stored at -20 °C or below, should be entirely thawed at room temperature before use.
- All reagents should be used immediately after thawing to reduce the time at room temperature.
- Vortex and spin-down steps are necessary when mixing the reagent.

9.2 PCR Master mix

① Prepare the PCR Master mix

Negative Control (NC) consisting of RNase-free water is required for the extraction and pollution monitoring of the RT-PCR process and is used throughout the sample processing procedure. Each run must include at least one negative control.

Positive Control (PC) consisting of PC (SCV2) is necessary to monitor that instruments and devices are functioning properly and being used throughout the sample processing procedure. Each run must include at least one positive control.

The amount of PCR Master mix should be prepared by calculating the overage, corresponding to at least 1~2 reactions more than the number of test and control(PCR Positive and Negative Control)

Components	Volume of 1 Test
Reaction Mix (SCV2)	**4** µL
Enzyme Mix (SCV2)	**2** µL
Oligo Mix (SCV2)	**4** µL
RNase free Water	**7** µL
Total volume	**17** µL

② Vortex and centrifuge briefly the PCR Master mix

③ Add PCR master mix to PCR tubes and add 3 µL of the nucleic acid sample.

- Negative Control (NC): Add 3 µL of RNase free Water instead of the nucleic acid sample.
- Positive Control (PC): Add 3 µL of Positive Control instead of the nucleic acid sample.

Components	Volume of 1 Test
PCR Master mix	**17** µL
Nucleic acid sample , or RNase free Water for negative control , or PC(SCV2) for positive control	**3** µL
Total volume	**20** µL

④ Close the PCR tube cap or film, and centrifuge briefly. Then make sure the solution is collected at the bottom of the tube.

9.3 Set-up and Running of the Instrument

① Selection of fluorophores for each target

Instrument	Target gene			
	S	ORF8	N	IC(RNase P)
CFX 96	FAM	HEX	Texas Red	Cy5
ABI 7500(Fast)	FAM	JOE	Texas Red	Cy5

② PCR Condition

Step	PCR Condition		Cycle(s)
	CFX 96	**ABI 7500(Fast)**	
cDNA Synthesis	50℃ for 10 min	50℃ for 10 min	1
Enzyme activation	95℃ for 3 min	95℃ for 3 min	1
PCR Amplification	95 ℃ for 1 sec	95 ℃ for 10 sec	2
	60 ℃ for 20 sec	60 ℃ for 20 sec	
	95 ℃ for 1 sec	95 ℃ for 10 sec	40
	60 ℃ for 5 sec Collect the fluorescence data	60 ℃ for 30 sec Collect the fluorescence data	

10. Result Analysis

10.1 Threshold and Base line setting

Instrument	Threshold	Base start-end
CFX 96	S, ORF, IC(RNaseP): 300, N: 400	-
ABI 7500(Fast)	All target, 10,000	3-15

10.2 Interpretation criteria for quality control

Negative and positive control tests should be examined before interpretation of patient results. If the control test results are invalid, the patient results cannot be interpreted or reported. Retest according to 'Troubleshooting.' Control test results should be interpreted according to the criteria listed in the below table.

Control	Target gene(Fluorescence) and Ct Value				Interpretation
	S(FAM)	ORF8(HEX/JOE)	N(Texas Red)	IC(Cy5)	
PC(SCV2)	< 27	< 27	< 27	< 27	Valid
NC	≥ 37 or N/A	≥ 37 or N/A	≥ 37 or N/A	≥ 37 or N/A	Valid

10.3 Interpretation criteria for specimen

① Individual target gene Ct value

Target	Fluorescence	Ct value	Interpretation
S	FAM	< 37	Positive (+)
		≥ 37 or N/A	Negative (-)
ORF8	HEX/JOE	< 37	Positive (+)
		≥ 37 or N/A	Negative (-)
N	Texas Red	< 37	Positive (+)
		≥ 37 or N/A	Negative (-)
IC	Cy5	< 37	Positive (+)
		≥ 37 or N/A	Negative (-)

② Result Interpretation

Case	IC	S	ORF8	N	Interpretation	Comment
1	+/-	+	+	+	SARS-CoV-2 Detected	Result is valid. Whether or not IC is detected is not an essential condition for target positive determination. IC amplification may be inhibited or not confirmed by the dominant amplification of the target.
2	+/-	Two or one of three is +			SARS-CoV-2 Detected	Result is valid. Missing amplification of individual targets may be due to: • a sample at concentrations near or below the limit of detection of test. • a mutation in the corresponding target region, or other factors.
3	+	-	-	-	SARS-CoV-2 Not detected	Result is valid.
4	-	-	-	-	Invalid/ Retest	Result is invalid. Repeat the test. If the result is still invalid, It is recommended to perform nucleic acid extraction again or obtain a new specimen.

11. Troubleshooting

11.1 If all the target signals and IC signal are not observed:

Probable causes	Solution
Error in specimen collection	Recollect the specimen.
Error in nucleic acid extraction	Extract the nucleic acid from the specimen again.
Incorrect PCR cycle or machine temperature	Check the PCR conditions and repeat the PCR under the correct setting.
The fluorescence for data analysis does not comply with the protocol	Select the correct fluorescence for each target listed in the protocol.
Reagent malfunction	Check the storage conditions and the expiration date of the reagents and use new reagents.
Presence of inhibitor	Re-extract the nucleic acid or dilute the template nucleic acid in RNase free Water (10-100x).

11.2 If signals are observed in the negative control:

Probable causes	Solution
Cross contamination	Decontaminate all surfaces and instruments. Use filter tips during the entire procedure. Repeat the nucleic acid extraction with the new reagents.
Reagent contamination	Use new reagents

11.3 If no signal is observed in the positive control:

Probable causes	Solution
Reagent malfunction	Re-assay with a new product. Make sure the product is stored in the recommended conditions.
Incorrect PCR setting	Repeat the PCR with corrected setting
The fluorescence for data analysis does not comply with the protocol	Select the correct fluorescence for each target listed in the protocol.
Incorrect PCR mixture	Check whether all components are correctly added.

12.Performance Characteristics

12.1 Analytical sensitivity

The study of analytical sensitivity (Limit of Detection; LoD) established the lowest SARS-CoV-2 viral RNA concentration that consistently yielded a 95% positivity rate with '*DirectFast™* SARS-CoV-2 multiplex assay'. The tests were conducted on two different types of PCR instruments (CFX 96 and ABI 7500). The nucleic acids were extracted from a nasopharyngeal swab collection (in UTM), which spiked with SARS-CoV-2 genomic RNA (NCCP No. 43326) at various concentrations (from $1x10^0$ ~ $1x10^6$ copies/µL). The viral RNA was obtained from the National Culture Collection for Pathogen (NCCP) in the Republic of Korea. Twenty-four replicates for each concentration were tested, and resultantly the detection limits of S, N, and ORF8 genes were determined as ten(10) copies/µL for each.

Instrument	S gene(FAM)	ORF8(JOE/HEX)	N(Texas Red)
CFX 96	10 copies/µL	10 copies/µL	10 copies/µL
ABI 7500(Fast)	10 copies/µL	10 copies/µL	10 copies/µL

12.2 Analytical specificity: Cross-reactivity

For the evaluation of the analytical specificity of '*DirectFast™* SARS-CoV-2 multiplex assay', cross-reactivity tests were done with twenty different pathogenic microorganisms. The tests were repeated three times on each sample, and no positive signals were observed for all of the tested pathogens. The tested pathogenic microorganisms are listed below.

No.	Pathogen	No.	Pathogen
1	Human Coronavirus OC43	11	Respiratory syncytial virus subtype B
2	Human Coronavirus 229E	12	Human Rhinovirus type 16
3	Human Coronavirus NL63	13	Echovirus 5
4	Influenza A (H1N1)	14	Streptococcus pneumonia
5	Influenza B	15	Haemophilus Influenzae
6	Adenovirus	16	Klebsiella pneumoniae
7	Parainfluenza virus 1	17	Pseudomonas aeruginosa
8	Parainfluenza virus 2	18	Bordetella pertussis
9	Parainfluenza virus 3	19	Staphylococcus aureus
10	Respiratory syncytial virus subtype A	20	Mycobacterium tuberculosis

12.3 Analytical specificity: Interference reactivity

Interference tests were conducted on four substances (Hemoglobin, Mucin, Mupirocin, Universal Transport Medium [UTM]) considering the endogenous and exogenous factors of the specimen, and no interference reactions occurred.

12.4 Analytical Precision: Repeatability

Repeatability was assessed by testing for 10 days, 2 runs per day, 2 replicates per run, 2 lot. Targets were using 2 levels of concentration. As a result, the detectability of samples showed 100%.

Instrument		CFX 96			ABI 7500(Fast)		
Target	copies/ µL	SD	CV (%)	Detectability (%)	SD	CV (%)	Detectability (%)
S	100	0.71	2.30	100	0.70	2.26	100
	10	1.07	3.15	100	1.06	3.12	100
ORF8	100	0.51	1.62	100	0.62	1.98	100
	10	0.66	1.88	100	0.68	1.93	100
N	100	0.66	2.20	100	0.71	2.38	100
	10	1.09	3.24	100	1.07	3.20	100

12.5 Analytical Precision: Reproducibility

Reproducibility study was performed with 2 different conditions: between-tester (3 testers), between-instrument(3 instruments). All results showed 100% agreements.

① Between- tester

-. CFX 96

Target	copies/ µL	Tester 1			Tester 2			Tester 3			Between-tester		
		SD	CV (%)	Detection (%)	SD	CV (%)	Detection (%)	SD	CV (%)	Detection (%)	SD	CV (%)	Detection (%)
S	100	0.62	2.00	100	0.80	2.58	100	0.72	2.33	100	0.71	2.29	100
	10	0.83	2.46	100	0.87	2.57	100	1.05	3.12	100	0.91	2.70	100
ORF8	100	0.63	2.00	100	0.55	1.74	100	0.46	1.46	100	0.54	1.73	100
	10	0.54	1.53	100	0.48	1.36	100	0.65	1.87	100	0.56	1.58	100
N	100	0.70	2.33	100	0.65	2.18	100	0.69	2.31	100	0.67	2.25	100
	10	0.74	2.22	100	0.82	2.44	100	0.79	2.36	100	0.78	2.31	100

-. ABI 7500(Fast)

Target	copies/ µL	Tester 1			Tester 2			Tester 3			Between-tester		
		SD	CV (%)	Detection (%)	SD	CV (%)	Detection (%)	SD	CV (%)	Detection (%)	SD	CV (%)	Detection (%)
S	100	0.67	2.16	100	0.83	2.68	100	0.68	2.22	100	0.72	2.34	100
	10	0.81	2.40	100	1.04	3.07	100	0.89	2.65	100	0.91	2.69	100
ORF8	100	0.50	1.59	100	0.57	1.80	100	0.64	2.02	100	0.57	1.81	100
	10	0.60	1.72	100	0.56	1.59	100	0.54	1.53	100	0.56	1.60	100
N	100	0.62	2.09	100	0.81	2.74	100	0.60	2.03	100	0.68	2.30	100
	10	0.77	2.30	100	0.87	2.61	100	0.87	2.62	100	0.83	2.50	100

② Between- instrument

-. CFX 96

Target	copies/ µL	Instrument 1			Instrument 2			Instrument 3			Between-instrument		
		SD	CV (%)	Detection (%)	SD	CV (%)	Detection (%)	SD	CV (%)	Detection (%)	SD	CV (%)	Detection (%)
S	100	0.74	2.40	100	0.79	2.57	100	0.72	2.34	100	0.74	2.41	100
	10	0.71	2.10	100	0.90	2.67	100	0.88	2.62	100	0.83	2.47	100
ORF8	100	0.64	2.04	100	0.56	1.78	100	0.60	1.90	100	0.60	1.89	100
	10	0.62	1.76	100	0.53	1.50	100	0.62	1.77	100	0.58	1.66	100
N	100	0.71	2.37	100	0.65	2.17	100	0.71	2.36	100	0.68	2.28	100
	10	0.78	2.31	100	0.78	2.33	100	0.73	2.19	100	0.76	2.26	100

-. ABI 7500(Fast)

Target	copies/ µL	Instrument 1			Instrument 2			Instrument 3			Between-instrument		
		SD	CV (%)	Detection (%)	SD	CV (%)	Detection (%)	SD	CV (%)	Detection (%)	SD	CV (%)	Detection (%)
S	100	0.68	2.21	100	0.68	2.21	100	0.73	2.39	100	0.69	2.25	100
	10	0.82	2.42	100	0.70	2.06	100	0.81	2.38	100	0.77	2.27	100
ORF8	100	0.55	1.76	100	0.56	1.77	100	0.54	1.71	100	0.54	1.73	100
	10	0.58	1.66	100	0.60	1.71	100	0.55	1.58	100	0.57	1.63	100
N	100	0.75	2.51	100	0.69	2.33	100	0.62	2.09	100	0.68	2.29	100
	10	0.80	2.39	100	0.85	2.55	100	0.64	1.90	100	0.76	2.27	100

13. References

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- Li Q, Guan X, Wu P et al. Early Transmission Dynamics in Wuhan, China, of Novel Coronavirus-Infected pneumonia. NEJM, 2020 Jan 29. DOI: 10.1056/NEJMoa2001316.
- W. Guan et al. Clinical Characteristics of Coronavirus Disease 2019 in China. N ENGLJ MED NEJM.org. 2020. DOI: 10.1056/NEJMoa2002032.

14. Technical Support

- For Technical support, please contact our dedicated technical support team on:

  * Phone: 82-42-867-9055
  * Email: info@nanohelix.net

LOT	Lot Number	REF	Catalogue Number
	Expiry date		Storage Temperature
	Caution	IVD	Reagent for *In Vitro* Diagnostic Analyzer
	Consult Instructions for use		Manufacturer
	Contains sufficient for tests	EC REP	Authorized representative in the European community
CE	This product fulfills the requirement of the European Directive 98/79/EC for *in vitro* diagnostic medical devices.		