Prepared in September 2021 (1st edition)

Instructions for Use
KANEKA RT-PCR Kit "SARS-CoV-2 (L452R/E484Q/E484K/N501Y)"

Precautions for use

- **Wear personal protective equipment (such as rubber gloves, safety glasses, and masks) before using KANEKA RT-PCR Kit.**
- **The results obtained from KANEKA RT-PCR Kit should be evaluated and used at your own discretion. Kaneka Corporation is not responsible, whether directly or indirectly, for any damages or loss which may be caused by the evaluation or use of the obtained results.**
- **You are responsible for verifying the validity of any detection results obtained using an operating procedure that is not indicated in these instructions for use.**
- **KANEKA RT-PCR Kit is a set of reagents intended for research applications and is not to be used to diagnose, or help to diagnose, a disease.**

1. Product description

KANEKA RT-PCR Kit is a set of reagents to detect L452R mutation, E484Q mutation, E484K mutation, and N501Y mutation, among mutations found in the SARS-CoV-2 spike protein, using 1-step RT-PCR (the reverse transcription polymerase chain reaction method) with fluorogenic probes.

The fluorogenic probes contained in the reaction solution are labelled with quencher on the 3' end and with fluorescent material (FAM for detecting L452R, Yakima Yellow™* for E484Q, ROX for E484K, and Cyanine 5 for N501Y) on the 5' end. During the process of DNA elongation that incorporates four types of deoxynucleotide triphosphate, the fluorogenic probes decompose and the fluorescent material previously quenched by the quencher is released, producing fluorescence. The fluorescence in each cycle of PCR is measured using a real-time PCR system, and the presence/absence of any SARS-CoV-2 spike protein gene variants is evaluated based on the Ct value of each fluorescence.

* "Yakima Yellow" is a trademark of Elitech Group.

2. Product structure/storage conditions

Component	Volume (100 tests)	Storage temperature	Expiration date
RT-PCR Enzyme Mix*1	600 µL x 1 vial	−10˚C to −30˚C	One year after manufacturing
L452R/E484Q/E484K/N501Y Primer & Probe Mix*2	500 µL x 1 vial		
RNase Free Water	1 mL x 1 vial		

*1. This mix contains enzymes, substrates, etc.

*2. Store this mix away from light because it contains fluorescent labeled probes.

3. How to use

3.1. Instruments and equipment that are necessary but not included in KANEKA RT-PCR Kit

- Micropipette and micropipette tips with filters
- Real-time PCR system (a system that can detect FAM, Yakima Yellow™, ROX and Cyanine 5)
- PCR tubes (0.2 mL)
- Desktop centrifuge
- Positive control (sold separately)

3.2. Preparation of samples (purification of RNA from the specimen)

The RNA extracted from the specimen is used as a sample in KANEKA RT-PCR Kit. Samples should be prepared according to "Manual for the Detection of Pathogen 2019-nCoV), published by the National Institute of Infectious Diseases.

3.3. Preparation of the master mix

(1) Thaw the RT-PCR enzyme mix completely by leaving it on ice. Thaw other reagents completely at room temperature (0˚C or higher). After thawing, mix each reagent thoroughly by inverting or pipetting, then spin down before opening the lid.

(2) Prepare the master mix according to the composition shown in the table below.

Master mix for one reaction

Reagent name	Additive amount
RT-PCR Enzyme Mix	6 µL
L452R/E484Q/E484K/N501Y Primer & Probe Mix	5 µL
RNase Free Water	9 µL
(Total amount)	20 µL

3.4. Preparation of the RT-PCR reaction solution

<Specimens>

Add 20 µL of the master mix to a tube containing the sample prepared in 3.2 and mix thoroughly by pipetting. *3

*3. Perform RT-PCR reaction immediately after preparing the reaction solution.

<Control reaction solution>

For proper evaluation of the result, prepare reaction solutions to which the negative control, positive control 1 (N501Y/E484K), *4 or positive control 2 (L452R/E484Q) *5 has been added. (Prepare at least one of each solution for each PCR run.)

(1) Use the RNase Free Water supplied with the kit as the negative control, and use a solution prepared to 10^3 copies/µL from commercially available artificially synthesized RNA as positive controls 1 and 2.

(2) After adding 5 µL of the negative control or a positive control to a tube, add 20 µL of the master mix, and mix thoroughly by pipetting.

*4. We have confirmed that the following products can be used as positive control 1 (N501Y/E484K):
- SARS-CoV-2 Gamma Strain Positive Control RNA (NIHON GENE RESEARCH LABORATORIES, INC.)
- Twist Synthetic SARS-CoV-2 RNA Control 16 (B.1.351) (Twist Bioscience)

*5. We have confirmed that the following product can be used as positive control 2 (L452R/E484Q):
- Twist Synthetic SARS-CoV-2 RNA Control 18 (B.1.617.1) (Twist Bioscience)

3.5. RT-PCR reaction

Place the PCR tubes containing the reaction solutions prepared in 3.4 on the real-time PCR system and perform RT-PCR reaction with the settings listed in the table below.

To detect fluorescence, read the instructions for use of the real-time PCR system and select a channel that allows the measurement of the following fluorescence.

FAM: wavelength of maximum absorbance = 495 nm, wavelength of maximum emissions = 520 nm

Yakima Yellow™: wavelength of maximum absorbance = 525 nm, wavelength of maximum emissions = 549 nm

ROX: wavelength of maximum absorbance = 576 nm, wavelength of maximum emissions = 602 nm

Cyanine 5: wavelength of maximum absorbance = 649 nm, wavelength of maximum emissions = 666 nm

Step	Temperature	Reaction time	Number of cycles	Fluorescence detection
1	52˚C	300 sec	1	OFF
2	95˚C	20 sec	1	OFF
3	95˚C	5 sec	45	OFF
	60˚C	30 sec		ON

4. How to evaluate

After performing RT-PCR reaction, check the amplification curve and confirm that the analysis parameters have been properly programmed according to the instructions for use of the real-time PCR system, then calculate the Ct values. Evaluate the results according to the criteria listed in the table below.

Test result of the control reactions

	FAM (L452R detection system)	Yakima Yellow™ (E484Q detection system)	ROX (E484K detection system)	Cyanine5 (N501Y detection system)
Negative control*6	Not detected	Not detected	Not detected	Not detected
Positive control 1 (N501Y/E484K)	Not detected	Not detected	Ct ≤ 35*7	Ct ≤ 35*7
Positive control 2 (L452R/E484Q)	Ct ≤ 35*7	Ct ≤ 35*7	Not detected	Not detected

*6. Confirm that the negative control was not detected. If a Ct value is calculated, it means that there is a possibility of contamination, so decontaminate the instruments and the work environment and run the test again.

*7. Confirm that the Ct value of the positive control is 35 or less. If the Ct value is higher than 35 or is not detected, check the settings of the equipment and run the test again.

Test results of specimens

Ct value*8	Evaluation result
Not detected	Negative or below detection limit*9
< 42	Positive
≥ 42	Retest is recommended*10

*8. Some real-time PCR system models may not calculate the Ct value for some amplification curves correctly. Always check the shape of the amplification curves and, if necessary, manually set the settings according to the instructions for use of the real-time PCR system.

*9. Even when the result is determined to be below the detection limit, a low copy number of the novel coronavirus gene may be present.

*10. If the Ct value ≥ 42, a retest is recommended. If the same result is obtained after a retest, it is recommended to determine the result as positive.

Examples of test results and evaluation result

L452R (FAM)	E484Q (Yakima Yellow™)	E484K (ROX)	N501Y (Cyanine5)	Evaluation result
−	−	−	+	Alpha strain
−	−	+	+	Beta strain, Gamma strain
+	−	−	−	Delta strain
+	+	−	−	Kappa strain

(Positive: +, Negative: −)

5. Precautions for use

- Always follow the storage conditions and expiration date indicated in these instructions for use.
- The specifications of KANEKA RT-PCR Kit are subject to change without prior notice.
- Follow the instructions specified by the manufacturers of the instruments and equipment being used.
- Replace the micropipette tip after each use in order to prevent contamination. It is recommended to use micropipette tips with filters.

- Clean the experiment table, etc., before and after using KANEKA RT-PCR Kit with 0.55% sodium hypochlorite, DNA removal agent, ultraviolet (UV) light, or other methods.
- In order to prevent false results caused by contamination, it is recommended that the RNA extraction, preparation of the RT-PCR reaction solution, and amplification and detection using a real-time PCR system be done in separate areas. Do not open and close the reaction tubes after amplification.
- Before opening tubes containing reagents, RNA extract, etc., spin down the tubes in a desktop centrifuge or other equipment (to prevent the content from dispersing).
- If contamination is confirmed, clean the instruments and equipment used by following the methods specified by their manufacturers.
- Reagents should not be frozen and thawed more than 15 times.
- Dispose of KANEKA RT-PCR Kit, RT-PCR reaction solution, RNA extract, and other materials in accordance with the rules concerning waste in your region and institution while considering hygiene and environmental factors.
- We have confirmed the performance of KANEKA RT-PCR Kit with the following real-time PCR system. If you are using other models, you are responsible for verifying the validity of the detected results yourself.
  CronoSTAR® 96 Real-Time PCR System (4ch) (Takara Bio USA)
  LightCycler® 96 System (Roche Diagnostics K.K.)
  LightCycler® 480 System II (Roche Diagnostics K.K.)
  * "CronoSTAR" is a registered trademark of Takara Bio Inc.
  * "LightCycler" is a registered trademark of Roche Diagnostics K.K.

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