RiboNAT™ Rapid Sterility Test
Instruction Manual

1st edition (September 2025)

Contents

1. Overview

This product is designed for the rapid detection of microorganisms in target samples. The reagents used in this test are composed of the following three kits:

- RNA Isolation Kit 1: Activates microorganisms and renders contaminating nucleic acid incapable of PCR amplification.
- RNA Isolation Kit 2: Extracts and purifies RNA from microorganisms.
- Detection Kit: Detects purified RNA through One-step Reverse Transcription Real-time PCR (One-step RT-PCR).

The entire procedure consists of three steps, as shown in Figure 1. In Step 1, Activator Solutions activate microorganisms under both aerobic and anaerobic conditions and render DNA from dead cells and free DNA incapable of PCR amplification.
In Step 2, the Enzyme Mix is used to degrade the cell walls of fungi and bacteria, while the Proteinase K Solution digests proteins. The kit includes DNase, which is used during purification and extraction steps. This DNase is applied for two-stage DNA degradation treatment of the intermediate and final solutions. The RNA Isolation Kit 2 is based on a sodium iodide (NaI) method. It denatures proteins in the sample and increases the solubility of hydrophobic molecules, solubilizing the nucleic acids into the solution. Purification of total RNA is then performed using magnetic beads. All purification procedures are carried out at room temperature.
In Step 3, ribosomal RNA is detected using One-step RT-PCR based on the fluorescent probe method. The kit includes Internal Control RNA solution for the purpose of extraction control. By using the Internal Control RNA during the RNA extraction step, it is possible to monitor the extraction procedures.

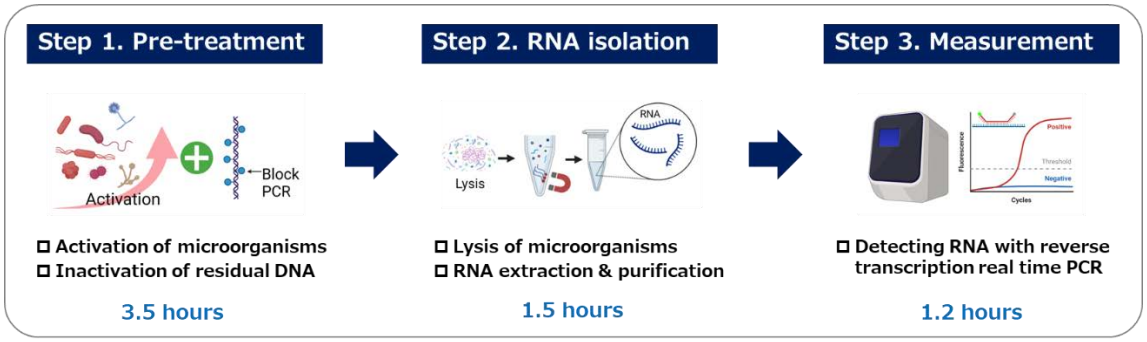

Figure 1. RiboNAT™Assay Flow

Illustrations were created with BioRender.

Features of RiboNAT™

- Extracts total RNA of microorganisms and detects ribosomal RNA.
- Wide range of bacteria and fungi can be detected with a single assay (qualitative test).
- Short time (7 hours) and high sensitivity (9 CFU/mL).
- Reduction of false positives.

2. Before use

- Please conduct experiments with attention to safety, in compliance with local regulatory requirements and in accordance with the rules of the experimental facility.
- Wear protective equipment such as gloves and protective glasses.
- Before starting the test, thoroughly clean the safety cabinet and pipettes with clean wipes containing RNase and nucleic acid removal reagent, ensuring the space is completely free of contamination by microorganisms or nucleic acids.
- When performing procedures, choose a workspace where the risk of contamination is as low as possible. RNA extraction operations should be conducted in a safety cabinet, and preparation of PCR plates should be carried out in a clean bench or safety cabinet in a sterile environment. If separate workspaces cannot be set for RNA extraction and PCR preparation, make sure not to cross-contaminate.
- Perform the tests using aseptic techniques.
- When placing hands or equipment inside the safety cabinet during operation, ensure that they are thoroughly cleaned with RNase and nucleic acid removal reagent beforehand.
- Do not unseal or open the reaction plate after completing RT-PCR. Opening the plate could significantly contaminate the environment with nucleic acids.
- An instructional video for the operating procedures is available. Please watch the video before starting the experiment.

3. Required kits and storage conditions

Code No.	Product name	Size	Storage
291-98401	RiboNAT™ Rapid Sterility Test - RNA Isolation Kit 1	50 Tests	-20°C
297-98001	RiboNAT™ Rapid Sterility Test - RNA Isolation Kit 2	50 Tests	Room temperature (≤ 30°C)
293-98101	RiboNAT™ Rapid Sterility Test - Detection Kit	100 Tests	-20°C

*1: All three products are required for the assay.

*2: Enzyme Enhancer, 1st-DNase Buffer (1) included in RNA Isolation Kit 1 and Water included in Detection Kit can be stored at room temperature.

<For cell suspension sample>

RiboNAT™ RNA Isolation Kits have been evaluated with samples of the following types of cells and concentrations. Different types of cells or larger concentrations may require optimization of the procedure.

- HEK293: 0.25 x 10^6 cells/mL (0.5 x 10^6 cells /2mL /assay)

- Mesenchymal stem cell (MSC)：0.5 x 10^6 cells/mL (1.0 x 10^6 cells/2mL/assay)
- T-cell：1.0 x 10^6 cells/mL (2.0 x 10^6 cells/2mL/assay)

4. Items required but not included in the kit

<Equipment>

- Benchtop centrifuge (1.5 mL and 0.2 mL tube)
- Block heater (37-95℃) and sockets: 15 mL, 1.5 mL, 0.2 mL tube
- Vortex Mixer
  This document provides specifications for the Vortex-Genie 2 from Electro Scientific Industries, Inc.
- Magnet stand (Fujifilm Wako #299-36421 or equivalent)
- Centrifuge and 1.5 mL tube rotor (centrifugal force required: 1,200 x g, 16,000 x g)
- Plate centrifuge (not necessary if the above centrifuge has a rotor for plate centrifugation)
- Incubator (60℃) or water bath (37℃)
- Real-Time PCR system (detection filters for 3 wavelengths are required)
  (1) 515-530 nm (representative dye: FAM), (2) 675-690 nm (representative dye: Cyanine 5),
  (3) 560-580 nm (representative dye: HEX or VIC)
  Ex.) - CFX96 System（Bio-Rad Laboratories, Inc）
  - Quant Studio® 5 Real-Time PCR System（Thermo Fisher Scientific Inc.）

< Pipettes and Consumables>

- Electronic pipette (Ex. Eppendorf Xplorer® 50-1000 µL, blue, #4861000040)
  This document provides specifications for the Eppendorf Xplorer®.
  4-channel type (Eppendorf Xplorer® plus Move It®, 4 channels, 50-1200µL, #4861000833) is recommended after gaining proficiency in operation.
- Electronic multi-dispenser pipette and tips (recommended)
  Ex.) Multipette® E3 (Eppendorf #4987000010)
  Combitips advanced 0.5 mL Biopur® (Eppendorf #0030 089 634)
  Combitips advanced 1.0 mL Biopur® (Eppendorf #0030 089 642)
  Combitips advanced 2.5 mL Biopur® (Eppendorf #0030 089 650)
  Combitips advanced 5.0 mL Biopur® (Eppendorf #0030 089 669)
- Micropipettes (2-200 µL, 100-1000 µL, 1- 10 mL)
- Pipette tips/ Nuclease-free/ filter (1-20 µL, 20-200 µL, 1000 µL, 10 mL)
- Real-Time PCR plates and plate seals
  Ex.) <For Bio-Rad Real-Time PCR device CFX96 >
  Plate: Hard-Shell® 96-Well PCR Plates (#HSP9645)
  Seal: Microseal 'B' PCR Plate Sealing Film (#MSB1001B)
  < For Thermo Fisher Scientific Real-Time PCR device QuantStudio®>

Plate: MicroAmp™ Optical 96-Well Reaction Plate (#N8010560）

Seal: MicroAmp™ Optical Adhesive Film (#4311971）

- RNase and nucleic acid removal reagent: RNase Knockout (Fujifilm Wako #181-03381) or Sterile ProChlor (Stabilised Hypochlorous acid in purified water) (Contec, Inc #SBT34PK)
- Clean wipes or equivalent

<Protective equipment>

- Disposable lab coats
- Disposable gloves
- Disposable mask
- Protective glasses
- Disposable cap

< Laboratory Facilities>

- Safety cabinet

  Recommended specification

  - JIS standard (JIS K3800) compliant model
  - Dust collection efficiency: 99.99% or higher for 0.3 μm particles (PAO)
- Room cleanliness: ISO class 7 or lower (recommended)

5. Kit components

RiboNAT™ Rapid Sterility Test - RNA Isolation Kit 1 (ik1)

Components	Cap color	Volume
Proteinase K Solution	Green	700 µL ×1
Enzyme Mix	Red	750 µL ×1
DNase Solution	Purple	380 µL ×1
1st-DNase Buffer (1)		23 mL ×1
2nd-DNase Buffer (2)	Yellow	180 µL ×1
Activator Solution 1 (SCDM)		50 mL ×1
Activator Solution 2 (AS)		50 mL ×2
Enzyme Enhancer		50 mL ×1
Nucleic Acid Inactivator		60 µL ×1

RiboNAT™ Rapid Sterility Test - RNA Isolation Kit 2 (ik2)

Components	Cap color	Volume
Lysis Buffer		6 mL ×1
Lytic Enhancer		3 mL ×1
Magnetic Beads	Black	1.5 mL ×1
Binding Buffer		16 mL ×1
Wash Buffer (A)		45 mL ×1
Wash Buffer (B)		45 mL ×1
Elution Buffer	Translucent	1.7 mL ×3
Sample Tube		50 pcs ×2
Elution Tube		7 pcs ×1

RiboNAT™ Rapid Sterility Test - Detection Kit (dk)

Components	Cap color	Volume
Detection Mix		1 mL ×2
Water	Blue	1 mL ×1
Positive Control RNA		400 µL ×1
Internal Control RNA	White	1 mL ×1

6. Test guideline

<Table 1. Test items>

Item	Description	Number of Extraction	Number of PCR wells
Samples to be tested	Perform RNA extraction and detection according to the protocol. (1mL each for aerobic and anaerobic culture)	1	2
Positive extraction control (PEC)	Add Internal Control RNA (IC) to all samples and perform RNA extraction and detection. [Purpose: Evaluate the efficiency of RNA extraction.]	-	-
Negative extraction control (NEC)	Add IC to unused medium or washing buffer and perform RNA extraction and detection. (1mL each for aerobic and anaerobic culture) [Purpose: Monitor for contamination during the extraction process.]	1	2
No-template control (NTC)	Dispense Water included in Detection Kit to 4 wells and perform PCR detection.	0	4
PCR positive control (PC)	Dispense Positive Control RNA included in Detection Kit to 4 wells and perform PCR detection.	0	4
Full run control* (optional)	Spike standard strain into samples and perform RNA extraction and detection. (1mL each for aerobic and anaerobic culture)	1	2
Total well			12*

*Full Run Control is optional. Please set it according to the test configuration.

7. Test procedure

- Please also refer to the instruction video before the experiment.
- (ik1), (ik2), and (dk) after the first mention of a reagent show which kit contains that reagent.
  - ik1: RNA Isolation Kit 1
  - ik2: RNA Isolation Kit 2
  - dk: Detection Kit

Part 1. Pre-treatment

<1-1: Medium preparation>

■ Thawing Activator Solutions

(1) Place Activator Solution 1 (SCDM) (ik1) (referred to as SCDM) and Activator Solution 2 (TG) (ik1) (referred to as TG) in an incubator at 60℃ for 30 minutes.

(2) After 30 minutes, mix them by inversion three times to check if they are fully thawed.

<Notes>

- If they are not fully thawed, place them again in the 60℃ incubator and check every 10 minutes to make sure they are completely thawed.
- If you do not have a 60℃ incubator, 37℃ water bath can be used as an alternative. In the case of using a water bath, place the bottles in a pouch or other container to avoid direct contact with the water to prevent contamination.

■ Preparation of Activator Solution 1 (SCDM)

(3) After thawing, cool on ice for at least 20 minutes.

(4) Divide the amount to be used of SCDM into a 15 mL centrifuge tube and add Nucleic Acid Inactivator (ik1) following the Table 2.

(5) Mix by inversion at least 3 times before use.

[Table 2. Mixing volume of SCDM and Nucleic Acid Inactivator]

	Number of samples			
	1	3	6	9
Activator Solution 1 (SCDM)	1.0 mL	3.0 mL	6.0 mL	9.0 mL
Nucleic Acid Inactivator	0.5 µL	1.5 µL	3 µL	4.5 µL

■ Preparation of Activator Solution 2 (TG)>

(6) After thawing, divide the amount to be used of TG into a 15 mL centrifuge tube.

(7) Heat at 95℃ for 10 minutes with the cap loosened (deaerating).

(8) Cool on ice for at least 20 minutes with the cap closed.

(9) Add Nucleic Acid Inactivator according to the Table 3 (*Do not add Nucleic Acid Inactivator before heating to 95°C). Mix by inversion at least 3 times before use.

*Unheated solution should be stored at -20°C.

[Table 3. Mixing volume of TG and Nucleic Acid Inactivator]

	Number of samples			
	1	3	6	9
Activator Solution 2 (TG)	1.7 mL	5.1 mL	10.2 mL	15.3 mL
Nucleic Acid Inactivator	0.5 µL	1.5 µL	3 µL	4.5 µL

*One 15 mL centrifuge tube is affordable up to 9 samples.

<1-2: Test sample preparation>

(10) Prepare two Sample Tubes (ik2) for each sample (for aerobic and anaerobic culture). Draw a line on the 0.2 mL mark of the Sample Tube with an oil-based pen (See Fig. 2).

(11) Vortex the sample to be tested and dispense 1 mL of sample into the marked tube.

(12) Set the tubes in the centrifuge rotor with the mark facing the inside (See Fig. 3).

(13) Centrifuge at 1,200 x g for 10 minutes at room temperature (20-27°C) to collect microorganisms.

(14) Slowly transfer the centrifuge rotor to a safety cabinet. After that, transfer the tubes slowly to the Magnet stand with the mark facing you. Using the Magnet stand is recommended to make operation (15) easier.

(15) On the Magnet stand, open the cap of the tubes and remove 0.8 mL of supernatant using an electronic pipette.

*Be careful not to put the tip below the mark to avoid aspirating microorganisms (See Fig.4). Use the slowest setting of the electronic pipette.

<Setting of the electronic pipette>

- Mode: Pip
- **Aspiration speed: 1 (slowest)**
- Dispense speed: 8 (fastest)

<1-3: Pre-culture>

(16) Add 1.5 mL of TG or 0.8 mL of SCDM to each sample tube. Mix by inversion 3 times after addition. *When using cell suspension samples, vortex until the cell clumps disperse.

(17) Incubate in a heat block at 37°C for at least 180 minutes. Turn off the light in the safety cabinet for light shielding.

(18) Prepare the Enzyme Mix (+) for use in Part 2. Add 8 µL of Nucleic Acid Inactivator (ik1) to the Enzyme Mix tube (ik1, Cap color: red). Incubate at 37℃ for 3 hours, then place on ice and keep on ice until use. After use, store at -20℃ or below. Before use, mix 10 times by inversion and spin down in a benchtop centrifuge. Once the Enzyme Mix (+) is prepared, there is no need to add more Nucleic Acid Inactivator until it is used up. (Nucleic Acid Inactivator is added to the Enzyme Mix only once.)

(19) After incubation, set the tubes in the centrifuge rotor with the mark facing the inside of the rotor.

(20) Centrifuge at 16,000 x g for 2 minutes at room temperature (20-27℃).

(21) Slowly transfer the centrifuge rotor into the safety cabinet. Transfer the tubes slowly to the Magnet stand with the mark facing you. Using the Magnet stand is recommended to make operation (22) easier.

(22) Remove 0.8 mL of supernatant from the SCDM-added Sample Tube and 1.5 mL from the TG-added Sample Tube using an electric micropipette.

*Be careful not to put the tip below the mark to avoid aspirating microorganisms (See Fig.4). Use the slowest setting of the pipette.

<Setting of electronic pipette>

- Mode: Pip
- **Aspiration speed: 1 (slowest)**
- Dispense speed: 8 (fastest)

(23) Aspirate the remaining solution in the SCDM-added Sample Tube 10 times, and then transfer the solution to the TG-added tube to combine them. When pipetting, note that microorganisms accumulate on the wall opposite of the mark. To collect the microorganisms, be sure to dispense the solution onto the wall opposite of the marker. (See Fig.4).

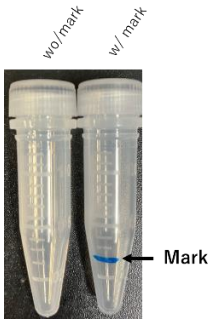

Figure 2.
Marking Sample Tube

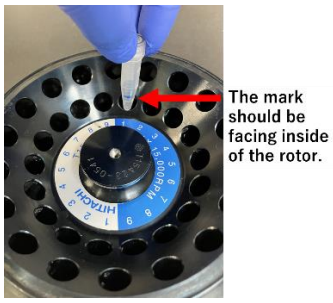

Figure 3.
Setting on the centrifugal rotor

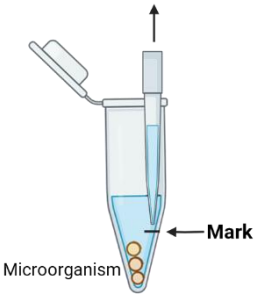

Figure 4.
Pellet of microorganisms

Part 2. RNA isolation

<2-1: Enzyme treatment>

(24) Add 1 mL of Enzyme Enhancer (ik1) to the sample (23) and mix by inversion three times.

(25) Put the tube into the centrifuge rotor with the mark facing the inside.

(26) Centrifuge at 16,000 x g for 2 minutes at room temperature (20-27°C).

(27) Remove 1.2 mL of supernatant with an electronic pipette. After removing 1.2 mL of supernatant, if the liquid level is above the 0.2 mL mark, remove the supernatant further down to the mark. Ensure that the final sample volume is between 200 µL and 250 µL.

*Be careful not to put the tip below the mark to avoid aspirating microorganisms (See Fig.4). Use the slowest setting for the electronic pipette.

<Setting of electronic pipette>

- Mode: Pip
- **Aspiration speed: 1 (slowest)**
- Dispense speed: 8 (fastest)

(28) Add 15 µL of Enzyme Mix (+) (Cap color: red) and vortex (Scale: 8) for 5 seconds.

(29) Spin down in a benchtop centrifuge.

(30) Incubate in a heat block at 37°C for 15 minutes.

(31) Add the following reagents in the order of (i), (ii), and (iii). *Do not pre-mix.

(i) Proteinase K (ik1, Cap color: green)	13.5 µL
(ii) Lysis Buffer (ik2)	120 µL
(iii) Lytic Enhancer (ik2)	60 µL

(32) Vortex (Scale: 8) for 5 seconds. Spin down in a benchtop centrifuge.

(33) Incubate in a heat block at 56°C for 20 minutes.

(34) During the incubation, prepare the DNase Mixture to be used in <2-2>. Mix the required volume of 1st-DNase buffer (1) (ik1) and DNase Solution (ik1, Cap color: purple) according to the Table 4 and mix by inversion at least 3 times. Keep the mixture at room temperature (20-27°C) until use.

[Table 4. Mixing volume of DNase Solution and 1st-DNase Buffer (1)]

	Number of samples							
	1	3	6	9	12	15	18	21
1st-DNase Buffer (1)	0.45 mL	1.4 mL	2.7 mL	4.1 mL	5.4 mL	6.8 mL	8.1 mL	9.5 mL
DNase Solution (Cap color: purple)	5.6 µL	17 µL	34 µL	50 µL	67 µL	84 µL	101 µL	118 µL

<2-2: RNA isolation>

(35) After 20 minutes incubation, add the following reagents in the order of (i), (ii), and (iii).

(i) Internal control RNA (dk, Cap color: white) *1 20 µL

(ii) Binding Buffer (ik2) 320 µL

(iii) Magnetic Beads (ik2, Cap color: black) *2 30 µL

*1. Thaw by placing at room temperature (20-27°C) for at least 5 minutes. After thawing, vortex (Scale: 8) for 10 seconds and mix thoroughly before use.

*2: Since Magnetic Beads tend to settle, invert the tube to mix and vortex (Scale: 10) for 20 seconds to ensure sufficient mixing just before use.

(36) Vortex (Scale: 8) for 10 seconds. *When using cell suspension samples, perform this step carefully as cells are likely to aggregate.

(37) Incubate at room temperature (20-27°C) for at least 5 minutes, allowing nucleic acids to bind to the Magnetic Beads.

(38) Spin down in a benchtop centrifuge.

(39) Place the tubes on the Magnet stand and let them sit for 30 seconds to allow the magnet beads to settle. Then, remove the solution.

(40) Remove the tubes from the Magnet stand and spin down in a benchtop centrifuge. This operation is performed to thoroughly remove the solution.

(41) Place the tubes on the Magnet stand, and after letting them sit for 15 seconds, remove the solution.

(42) Remove the Sample Tube from the Magnet stand, add 400 µL of DNase Mixture, aspirate with a pipette at least 4 times to disperse the Magnetic Beads, and place on a tube rack. Do not set the tube on the Magnet stand. *When using cell suspension samples, perform pipetting step more thoroughly.

(43) Let the tubes stand on the tube rack for 5 minutes or longer at room temperature (20-27°C).

(44) Place the tubes on the magnet stand, and after letting them sit for 30 seconds, remove the solution.

(45) Open the Magnet stand (See Fig.5) and add 900 µL of 1st-Wash Buffer (1) (ik2) with an electronic pipette (Mode: P/M), pipette at least 4 times to ensure sufficient dispersion.

<Setting of electronic pipette>

- Mode: P/M
- Aspiration speed: 4
- Dispense speed: 4
- Number of cycles: 4

(46) Close the Magnet stand (See Fig. 5), and after letting them sit for 30 seconds, remove the solution.

(47) Open the Magnet stand and add 900 µL of the 2nd-Wash Buffer (2) (ik2) using an electronic pipette, and pipette at least 4 times to ensure sufficient dispersion. Use the same pipette setting as (45).

(48) Close the magnet stand, and after letting the tubes sit for 30 seconds, remove the solution.

(49) Remove the tube from the magnet stand and spin down with a benchtop centrifuge, then set it on

the magnet stand again and remove the solution with a 200P pipette.

(50) Loosen the cap and let it stand on a heat block at 60℃ for 5 minutes to dry.

(51) Add 50 µL of Elution Buffer (ik2, Cap color: translucent) and vortex (Scale: 10) for 5 seconds.

(52) Place in a heat block at 60℃ for 5 minutes to elute nucleic acids from the magnetic beads.
Do not leave it for more than 10 minutes.

(53) After elution, spin down the tubes in a benchtop centrifuge, set them on the magnet stand, and after letting them sit for 30 seconds, collect the solution in the Elution Tube (ik2). This is the Extracted RNA solution.
*If stopping the procedure here, store the Extracted RNA solution at -80℃ or below.

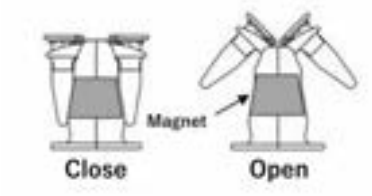

Figure 5: “Open” and “Close” position of Magnet stand.
(Example of Magnetic stand from Fujifilm Wako #299-36421).

Part 3. Measurement

<3-1: DNase reaction②>

(54) Add 1 µL of the 2nd-DNase Buffer (2) (ik1, Cap color: yellow) and 1 µL of the DNase Solution (ik1, Cap color: purple) to the Extracted RNA solution.
[When handling multiple samples, mix the 2nd-DNase Buffer (2) and the DNase Solution beforehand and add 2 µL of the mixture]

(55) Vortex (Scale: 8) for 5 seconds and spin down in a benchtop centrifuge.

(56) Heat them on a PCR device, (i) 37℃, 5 min → (ii) 65℃, 5 min → (iii) 4℃ 1 min or longer.
(Start the step (59) during this reaction time.)

(57) The solution after (56) is called the Final Processed RNA solution.

(58) Perform One-step RT-PCR immediately or store at -80℃ or below.

<3-2: Preparing assay plate>

(59) Thaw the Detection Mix (dk) at room temperature (20-27℃) for 10 minutes, mix by inversion 10 times, and confirm by pipetting that it is fully thawed before use.
*After use, store immediately in a -20℃ freezer. Do not leave it on ice or room temperature.

(60) Add 20 µL of the Detection Mix to each well of the Real-Time PCR plate.

(61) Add each solution according to the number in Fig. 6. Perform the measurements with N=2 for samples and NEC, and N=4 for NTC and PC.

- Detection Mix: 20 µL
- Sample: 10 µL Total 30 µL/well

								②			①	
	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample	Sample	Sample	Sample	Sample	Sample		NEC	NEC		NTC	NTC
B	Sample	Sample	Sample	Sample	Sample	Sample					NTC	NTC
C	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample				
D	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample				
E	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample			FRC	FRC ⑤
F	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample				
G	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample			PC	PC
H	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample			PC	PC
	③										④	

Figure 6: Example of plate layout

① No-template control (NTC): Water (dk, Cap color: blue) : 10 µL

② Negative extraction control (NEC) (Final processed RNA solution): 10 µL

③ Sample (Final processed RNA solution): 10 µL

④ PCR positive control (PC): Positive Control RNA (dk, Cap color: none) 10 µL

⑤ Full Run Control (FRC)

*Positive controls should be handled with care to avoid cross-contamination. In the example, well G11, G12, H11, and H12 in the front corner of the plate are used for PC.

*Full run control (FRC) is optional. If necessary, spike strain to the sample and perform the assay.

(62) Seal the plate with a plate seal, then vortex and spin down using a plate centrifuge.

<3-3: Measurement>

■ One-step RT-PCR conditions

1. 37℃, 5 min.
2. 95℃, 10 sec.
3. 65℃, 10 min.
4. 95℃, 10 sec.
5. 95℃, 10 sec.
6. 65℃, 30 sec. Fluorescent imaging: 37 cycles of 5-6.

*Temperature ramp rate: Default setting.

*For instruments such as the QuantStudio® series from Thermo Fisher Scientific, set ROX as the Passive reference.

■ Wavelength setting

[Table 5. Detection wavelength for each target]

Detection Target	Wavelength (nm)	Target sequence	Representative dye
Bacteria	515 - 530	23S rRNA	FAM
Fungi/Mold	675 - 690	28S rRNA	Cyanine 5
Internal Control	560 - 580	Artifact RNA	VIC, HEX

8. How to judge the results.

Follow the chart to judge the results.

Step 1: Check if there are any issues for the detection reagents, plate, operation and testing environment.

☐ Sample type: NTC（N=4 well）

Detection channels	Evaluation criteria
Bacteria (FAM)	Ct＞37
Fungi/Mold (Cyanine 5)	Ct＞37
Internal Control (HEX or VIC)	Ct＞37

N=4/4 Yes →

No ↓

Test Failed
Contamination
→Repeat PCR
Confirm that the environment is sterile and take great care when preparing reagents for the PCR plate.

☐ Sample type: PC（N=4 well）

Detection channels	Evaluation criteria
Bacteria (FAM)	Ct＜16
Fungi/Mold (Cyanine 5)	Ct＜16
Internal Control (HEX or VIC)	Ct＜16

N=4/4 Yes →

Evaluation items	Evaluation results
RT-PCR performance	Passed
Plate	Passed

No ↓

Test Failed
Reagent preparation errors
→Repeat PCR
Check the expiration date, storage temperature, and whether the reagent has been mixed before use.

Step 2: Check if there are any issues for the RNA extraction reagents, operation and testing environment.

☐ Sample type: NEC（N=2 well）

Detection channels	Evaluation criteria
Bacteria (FAM)	Ct＞35
Fungi/Mold (Cyanine 5)	Ct＞35
Internal Control (HEX or VIC)	Ct≦35

N=2/2 Yes →

Evaluation items	Evaluation results
RNA extract performance	Passed
Handling techniques, operating environment	Passed
Suitability of negative determination for samples	Passed

N=0/2, No ↓

N=1/2 Indeterminate → Repeat RT-PCR

Test Failed
If the Ct value for bacteria and fungi/mold is 35 or less, contamination is present.
→ Repeat RNA extraction
Check the sterile environment and reagents for possible contamination with nucleic acids and microorganisms.

Step 3: Positive/Negative judgement for the sample to be tested.

☐ Sample type: Specimen to be tested (Unknown sample)

Tests are valid

Detection channels	Evaluation criteria
Bacteria (FAM)	Ct≦35
Fungi/Mold (Cyanine 5)	Ct＞35
Internal Control (HEX or VIC)	Ct≦35

Yes → Bacteria Positive

Detection channels	Evaluation criteria
Bacteria (FAM)	Ct＞35
Fungi/Mold (Cyanine 5)	Ct≦35
Internal Control (HEX or VIC)	Ct≦35

Yes → Fungi/Mold Positive

Detection channels	Evaluation criteria
Bacteria (FAM)	Ct≦35
Fungi/Mold (Cyanine 5)	Ct≦35
Internal Control (HEX or VIC)	Ct≦35

Yes → Bacteria Positive
Fungi/Mold Positive

Detection channels	Evaluation criteria
Bacteria (FAM)	Ct＞35
Fungi/Mold (Cyanine 5)	Ct＞35
Internal Control (HEX or VIC)	Ct≦35

Yes → Negative
Below detection limit of this test

No ↓

Detection channels	Evaluation criteria
Bacteria (FAM)	Ct＞35
Fungi/Mold (Cyanine 5)	Ct＞35
Internal Control (HEX or VIC)	Ct＞35

Yes → Test Failed
If the Ct value for bacteria, fungi/mold and Internal Control are larger than 35, RNA extraction and purification process is failed.
→Repeat RNA extraction. Please check the test method.

Detection channels	Evaluation criteria
Bacteria (FAM)	Ct≦35
Fungi/Mold (Cyanine 5)	Ct≦35
Internal Control (HEX or VIC)	Ct＞35

Yes → Test Failed
Probably positive, but it is suspected that the test was not performed properly.
→ Repeat RNA extraction. Please check the test method.

*When using Quant Studio® series, set the Threshold value as follows.

- FAM: 0.8,　- Cy5: 0.8,　- VIC: 0.2

*2 wells should be loaded for each extraction sample. If the results are not the same in both wells, perform RT-PCR again. If the results are still not qualified, perform the test again from the beginning.

9. Precautions

<For the judgment>

Criteria are set for the following Real-Time PCR devices. Since each Ct value varies depending on the Real-Time PCR system, please verify the Ct value with your instrument. Please contact us for the validation method.

■ Validated systems

- Quant Studio® 5 Real-Time PCR System from Thermo Fisher Scientific Inc.
- CFX96 System from Bio-Rad Laboratories, Inc.

<For disposal>

When microorganisms are added to samples for validation, autoclave the used sample tubes and consumables to sterilize them. After the PCR reaction, do not unseal the PCR plate. Disposal should be in accordance with the applicable regional laws and regulations as well as rules in the organization.

10. Preparation of microorganism spike samples

This section describes how to prepare the microorganism spike samples for validation and performance tests. When preparing solutions with each strain's concentration, do not perform serial dilutions, instead, directly prepare solutions with the specified concentration. Here, we describe an example of how to prepare 3, 9 and 99 CFU/mL solutions using BIOBALL® from BioMérieux S.A. In-house strains can be used. Examples of commercially available standard strains are listed below.

- EZ-Accu Shot™, EZ-CFU™ One Step, etc. form Microbiologics, Inc.
- BIO-BALL® from BioMérieux S.A.

<Preparation method of spiked sample>

1. Add the required amount of sample to a centrifuge tube.
2. Add the required number of strain (See tables) to the tube.
3. Let the tube stand for 30 seconds, then vortex (Scale: 8) for 5 seconds.
   This is the microorganism spiked sample.
   *If not used immediately, vortex (Scale 8) for 2 seconds just before use, then dispense into the Sample Tube.

<Examples of combinations for testing>

Combination (1) *Candida albicans, Bacillus subtilis subsp spizizenii*

Combination (2) *Clostridium sporogenes*, *Aspergillus brasiliensis*

Combination (3) *Staphylococcus aureus*

Combination (4) *Pseudomonas aeruginosa*

Pseudomonas aeruginosa easily cause cross-contamination and should be handled with care.

■ Table 6. Examples of preparations with 3 CFU/mL strain solution

Number of tests	1	2	3	4	5	6	7	8
Required volume (mL)	2.2	4.4	6.6	8.8	11	13.2	15.4	17.6
BIOBALL 30 CFU (pcs)	1	1	1	1	2	2	2	2
Inoculation strain amount (CFU)	30	30	30	30	60	60	60	60
Samples (mL)	9.8	9.8	9.8	9.8	20	20	20	20
Concentration (CFU/mL)	3	3	3	3	3	3	3	3
Centrifuge tube size (mL)	15	15	15	15	50	50	50	50

■ Table 7. Examples of preparations with 9 CFU/mL strain solution

Number of tests	1	2	3	4	5	6	7	8
Required volume (mL)	2.2	4.4	6.6	8.8	11	13.2	15.4	17.6
BIOBALL 30 CFU (pcs)	1	2	3	3	4	5	5	6
Inoculation strain amount (CFU)	30	60	90	90	120	150	150	180
Samples (mL)	3.3	6.6	9.9	9.9	13.3	16.7	16.7	20.0
Concentration (CFU/mL)	9	9	9	9	9	9	9	9
Centrifuge tube size (mL)	15	15	15	15	50	50	50	50

■ Table 8. Examples of preparations with 99 CFU/mL strain solution

Number of tests	1	2	3	4	5	6	7	8
Required volume (mL)	2.2	4.4	6.6	8.8	11	13.2	15.4	17.6
BIOBALL 550 CFU (pcs)	1	1	2	2	3	3	3	4
Inoculation strain amount (CFU)	550	550	1100	1100	1650	1650	1650	2200
Samples (mL)	5.6	5.6	11.1	11.1	16.7	16.7	16.7	22.2
Concentration (CFU/mL)	99	99	99	99	99	99	99	99
Centrifuge tube size (mL)	15	15	15	15	50	50	50	50

11. Instruction video

Please access it directly via the link or QR code below, or search by product code number on our website (https://labchem-wako.fujifilm.com).

https://labchem-wako.fujifilm.com/jp/product/detail/W01W0129-9840.html

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<Checklist of the criteria>

No.	**Step 1: Determine the Validity of PCR, Plate, Procedure, and Test Environment**	Detection dye	N=1：Ct or Cq	N=2：Ct or Cq	N=3：Ct or Cq	N=4：Ct or Cq	Criteria	Check
1	Measure Water in 4 wells and confirm that the Ct or Cq values of all 3 wavelengths in all 4 wells meet the criteria.	FAM					Ct＞37	☐
		Cyanine 5					Ct＞37	☐
		HEX or VIC					Ct＞37	☐
2	Measure the Positive Control in 4 wells and confirm that amplification occurs for all 3 wavelengths in all 4 wells and that the Ct or Cq values meet the criteria.	FAM					Ct＜16	☐
		Cyanine 5					Ct＜16	☐
		HEX or VIC					Ct＜16	☐

No.	**Step 2: Verify the Extraction Reagent, Procedure, and Test Environment**	Detection dye	N=1：Ct or Cq	N=2：Ct or Cq	N=3：Ct or Cq	N=4：Ct or Cq	Criteria	Check
3	Measure the Negative Extraction Control (NEC) in 2 wells and confirm that the values of all 2 wells meet the criteria.	FAM			[redacted]		Ct＞35	☐
		Cyanine 5					Ct＞35	☐
		HEX or VIC					Ct≦35	☐

					Criteria			
No.	**Step 3: Evaluate the Measurement Target for Positive or Negative**	Detection dye	N=1：Ct or Cq	N=2：Ct or Cq	☐Bacteria positive	☐Fungi/mould positive	☐Bacteria &fungi/mould positive	☐Negative
4	Measure the sample in 2 wells and make judgement based on the Ct or Cq values of these 2 wells.	FAM			Ct≦35	Ct＞35	Ct≦35	Ct＞35
		Cyanine 5			Ct＞35	Ct≦35	Ct≦35	Ct＞35
		HEX or VIC			Ct≦35	Ct≦35	Ct≦35	Ct≦35