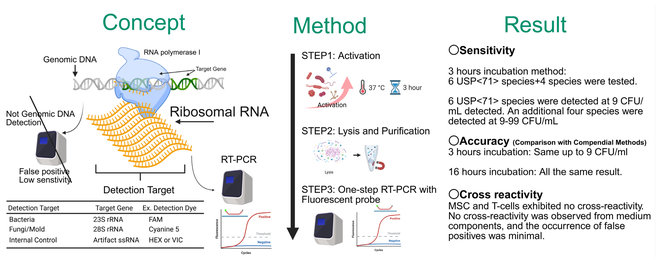
Rapid Sterility Testing by NAT Method Targeting RNA Instead of DNA

[1]Yotaro Yamamoto, [1]Rikiya Kanda, [1]Yukiko Inui, [2]Michiyo Nakamura, [1]Tomohiro Mori, [1]Takahiro Nishibu
[1]FUJIFILM Wako Pure Chemical Corporation R&D Marketing Operations Biotechnology Center
[2]FUJIFILM Wako Pure Chemical Corporation R&D Marketing Operations QC Product Development Department

Abstract

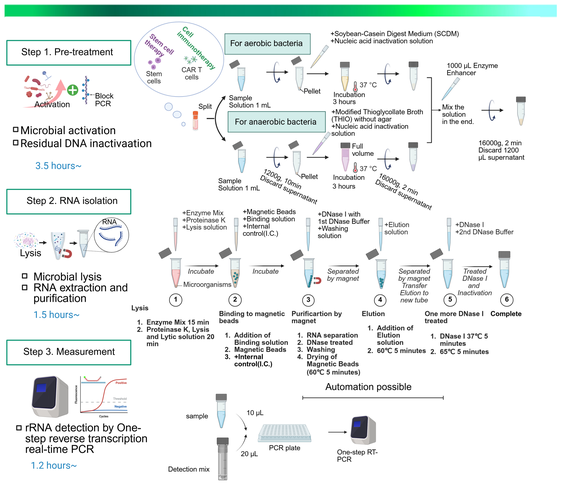
Concept: This study demonstrated a method for detecting ribosomal RNA (rRNA) as a rapid screening for bacterial and/or fungal/mold contamination.
Method: Conducted in three steps. 1st STEP: Microbial activation and inactivation of free nucleic acids and nucleic acids from dead microorganisms. 2nd STEP: Lysis using cell wall-degrading enzymes, surfactants, and protease K, followed by purification with magnetic beads. 3rd STEP: Detection via one-step reverse transcription PCR.
Results: Rapid detection of the six microbial species listed in USP Chapter <71> plus four additional species was achieved. By increasing the incubation time, sensitivity equivalent to conventional methods could also be attained. No cross-reactions were observed with various cells or culture media. These findings demonstrate that rapid microbial detection is possible by combining short incubation times with RNA detection.



Introduction

Real-time PCR is a promising detection method for rapid sterility testing due to its fast turn around time. However, challenges such as decreased sensitivity and false positives caused by DNA contamination from environmental sources and dead microorganisms remain. To address these issues, we developed a novel method targeting transcribed rRNA instead of conventional genomic DNA.

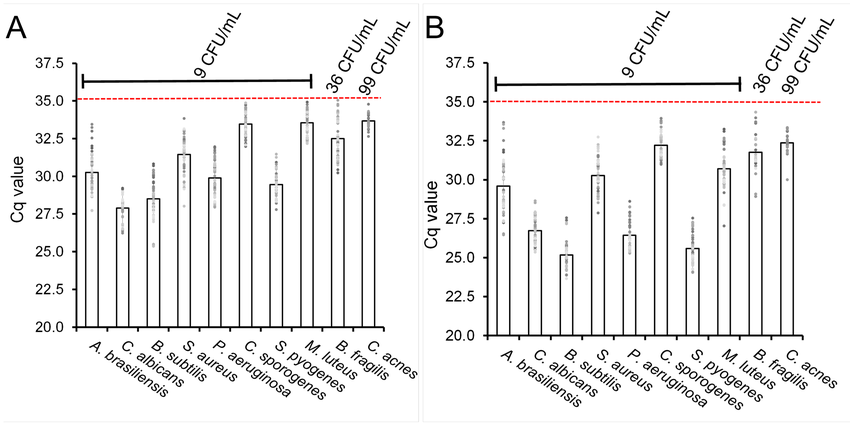
Method -Overview of RiboNAT™ Assay Flow-



Result

Some of the icons in this document were created using BioRender.

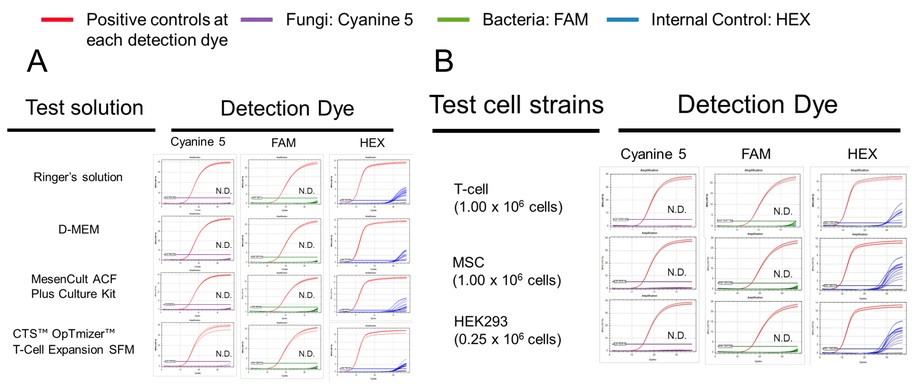
Figure 1 -Limit of Detection, 3 hours incubation-



A: This bar graph illustrates the average Cq values for each bacterium at the limit of detection (LOD) over 24 repeated tests following a 3-hour incubation. Each dot represents an individual data point. The test cutoff at Cq 35 is marked by the red dashed line.
B: This bar graph presents the average Cq values from repeated tests conducted with 0.5 × 10^6 T-cells per preparation after a 3-hour incubation. Individual data points are also displayed.
The number of assay: 24 times (One assay = RNA extraction: 1 time → RT-PCR: 2 wells)
Total：48 wells

The LOD of RiboNAT™ standard protocol (3 hours of pre-culture) for the six microorganisms specified in the pharmacopeias (JP, USP, EP), *Streptococcus pyogenes* and *Micrococcus luteus* was 9 CFU/mL. The LOD for *Bacteroides fragilis* and *Cutibacterium acnes* included in EP 2.6.27 were 36 and 99 CFU/mL, respectively. The results remained consistent even in cell suspension sample preparations.

Figure 3 -Cross reactivity test-

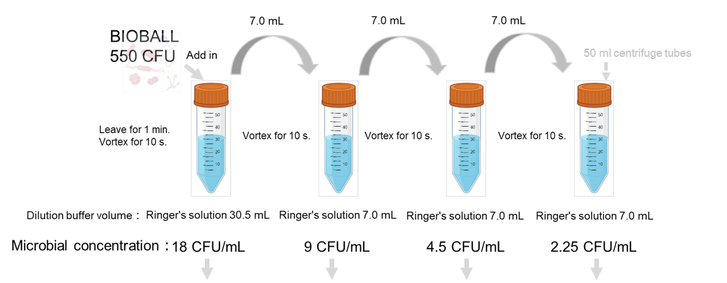


A: This panel shows the RT-PCR amplification curves for tests conducted with four types of solutions (culture mediums and cell washing solution). In each plot, the X-axis represents the number of cycles, and the Y-axis represents the fluorescence intensity.
B: This panel displays the RT-PCR amplification curves for tests using a fixed number of cells from three cell types: Mesenchymal Stem Cells (MSCs), Human Peripheral Blood Pan-T Cells (T-cells), and Human Embryonic Kidney 293 cells (HEK293).

**Internal controls were successfully detected, and no amplification was observed at the bacterial and fungal detection wavelengths.
No cross-reactivity was observed.**

Figure 2 -Accuracy, Comparison between compendial methods and RiboNAT™-

A



White cell: Positive
Gray cell: Negative

B

		RiboNAT Sterity test								Compendial method			
		3 hour				16 hour				14 day			
Microorganism name	Strain name	2.2 CFU/mL	4.5 CFU/mL	9 CFU/mL	18 CFU/mL	2.2 CFU/mL	4.5 CFU/mL	9 CFU/mL	18 CFU/mL	2.2 CFU/mL	4.5 CFU/mL	9 CFU/mL	18 CFU/mL
A. brasiliensis	NCPF2275	−(1/3) Cq: 34.1	−(1/3) Cq: 32.8	+(3/3) Cq: 32.4	+(3/3) Cq: 30.1	+(3/3) Cq: 20.9	+(3/3) Cq: 19.2	+(3/3) Cq: 18.3	+(3/3) Cq: 17.2	+(3/3) 9 day	+(3/3) 9 day	+(3/3) 9 day	+(1/3, 2/3) 9 day, 6 day
B. subtilis	NCTC10400	+(3/3) Cq: 30.7	+(3/3) Cq: 31.4	+(3/3) Cq: 28.9	+(3/3) Cq: 27.7	+(3/3) Cq: 12.1	+(3/3) Cq: 13.1	+(3/3) Cq: 12.3	+(3/3) Cq: 14.4	+(3/3) 3 day	+(3/3) 3 day	+(3/3) 3 day	+(3/3) 3 day
C. albicans	NCPF3179	+(3/3) Cq: 29.6	+(3/3) Cq: 27.9	+(3/3) Cq: 27.0	+(3/3) Cq: 25.4	+(3/3) Cq: 17.7	+(3/3) Cq: 17.6	+(3/3) Cq: 15.4	+(3/3) Cq: 14.3	+(3/3) 9 day	+(3/3) 9 day	+(3/3) 9 day	+(3/3) 9 day
P. paraeruginosa	NCTC12924	−(2/3) Cq: 34.2	+(3/3) Cq: 32.9	+(3/3) Cq: 31.3	+(3/3) Cq: 29.6	+(3/3) Cq: 10.1	+(3/3) Cq: 7.5	+(3/3) Cq: 7.8	+(3/3) Cq: 7.8	+(3/3) 1 day	+(3/3) 1 day	+(3/3) 1 day	+(3/3) 1 day
S. aureus□	NCTC10788	−(1/3) Cq: 35.2	−(1/3) Cq: 35.3	+(3/3) Cq: 32.6	+(3/3) Cq: 31.0	+(3/3) Cq: 10.3	+(3/3) Cq: 11.0	+(3/3) Cq: 11.1	+(3/3) Cq: 9.9	+(3/3) 2 day	+(3/3) 2 day	+(3/3) 2 day	+(3/3) 2 day
C. sporogenes	NCTC12935	−(0/3) Cq: 35.3	−(0/3) Cq: 35.1	+(3/3) Cq: 34.1	+(3/3) Cq: 33.3	+(3/3) Cq: 20.7	+(3/3) Cq: 19.0	+(3/3) Cq: 15.0	+(3/3) Cq: 14.3	+(3/3) 1 day	+(3/3) 1 day	+(3/3) 1 day	+(3/3) 1 day
Ringer's solution		−(3/3)				−(3/3)				−(3/3)			

A: The sample preparation method and an overview of the tests comparing RiboNAT™ and compendial methods are shown. Tests with N=3 were conducted for each prepared concentration. The tests were performed using the strains listed in USP <71>.
B: This table summarizes the detection results for RiboNAT™, including the corresponding Cq values for 3-hour and 16-hour incubation periods. For the conventional method, the table shows whether detection was achieved and the number of days required for detection.

☐RiboNAT™ -3 hours incubation-
- All 6 strains were detected at 9 CFU/mL.
- Two strains were detected at 2.25 CFU/mL.
☐ RiboNAT™ -16 hours incubation-
- Detected all 6 strains at 2.25 CFU/mL

☐Compendial method -14 days culture-
- Detected all 6 strains at 2.25 CFU/mL.

Conclusion

The rRNA detection method used in this study successfully detected bacteria and fungi in the presence of cells and culture medium. Over a 3-hour incubation period, the six species listed in USP <71> were detected at 9 CFU/mL, while an additional four species were detected at 9–99 CFU/mL. Comparison with compendial method demonstrated that equivalent sensitivity could be achieved by optimizing the incubation time. No cross-reactivity with cells or medium components was observed, indicating a significant reduction in false positives. By targeting rRNA, which reflects microbial viability, this method offers accurate sterility assessments in a substantially shorter timeframe compared to culture-based methods.

Acknowledgements

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