Cat. # CY232

For Research Use

TaKaRa

CycleavePCR™ Mycoplasma Detection Kit

Product Manual

v201903Da

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I. Description

The Cycleave PCR Mycoplasma Detection Kit enables the specific detection of *Mycoplasma* using real-time PCR amplification of the *Mycoplasma* 16S rRNA gene. Detection of at least ten species in the genus *Mycoplasma* (including species found in cell culture media: *M. arginini, M. hominis, M. hyorhinis, M. orale, M. salivarium, M. fermentas, M. bovis, M. arthritidis, M. pirum, and M. pneumoniae)* and one species in the genus *Acholeplasma* (*A. laidlawii*) has been confirmed.

For PCR amplification, this kit uses *TaKaRa Ex Taq*® HS, a hot-start PCR enzyme that prevents non-specific amplification from mispriming or primer dimer formation during reaction mixture preparation or other pre-cycling steps, and allows high-sensitivity detections.

This kit detects amplification products by cycling probe technology, which provides highly sensitive detections through the combined use of an RNA/DNA chimeric probe and RNase H. This method enables efficient detection of specific sequences of the gene fragments during PCR amplification. One end of the probe is labeled with a fluorescent moiety and the other end with a quencher. When intact, this probe does not emit fluorescence due to the action of the quencher. However, when the probe forms a hybrid with the complementary sequence of an amplification product, RNase H cleaves RNA in the chimeric probe, resulting in strong fluorescent signal emission (Figure 1). The amount of amplified product can be monitored by measuring the intensity of emitted fluorescence.

With this method, any RNA/template mismatch will prevent RNase H cleavage and will not be detected. The probe in this kit is perfectly complementary to *Mycoplasma* 16S rRNA, and therefore, *Mycoplasma* spp. will be detected specifically with high sensitivity (see Section VIII. Appendix, Table 1 for sequence information).

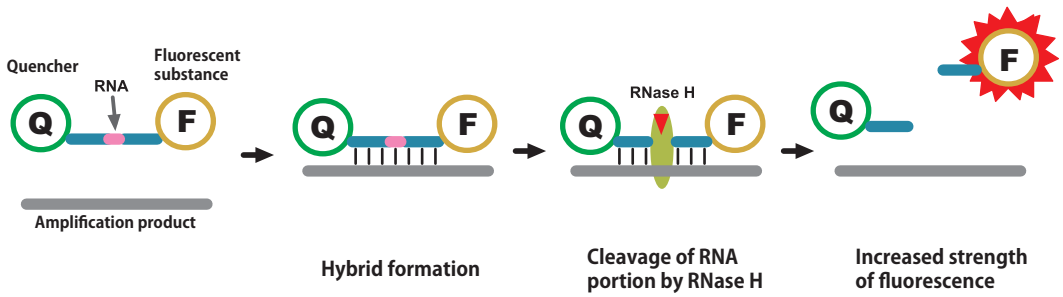


Figure 1. Principle of cycling probe technology.

II. Components (25 μl reaction volume, 50 reactions)

1.	2X CycleavePCR Reaction Mix	625 μl
2.	Myco. Primer/Probe Mix (5X conc.)	250 μl ＊1
3.	RNase Free dH_2O	1 ml
4.	Myco. Positive Control (1 x 10^6 copies/μl)	20 μl ＊2
5.	Proteinase K	50 μl

＊1 Store protected from light. This component contains a fluorescently labeled probe.

＊2 Any accidental contamination of any one of components 1 through 3 will cause inaccurate results. Use the small box supplied with the kit for storing this component separately from the others.

[Component information]

2X CycleavePCR Reaction Mix:
A PCR reaction reagent containing enzymes, buffer, and dNTP mixture.

Myco. Primer/Probe Mix:
A primer/probe mixture for detecting *Mycoplasma* containing specific primers to amplify the 16S rRNA gene of *Mycoplasma* and the Myco. Positive Control. A FAM-labeled probe detects the 16S rRNA gene of *Mycoplasma*, and a ROX-labeled probe detects the Myco. Positive Control.

RNase Free dH_2O:
Use this component to prepare the reactions.

Myco. Positive Control:
A positive control used to check for the presence of PCR reaction inhibitors. For PCR products amplified with Myco. Primer using Myco. Positive Control as the template, detection is possible with either the FAM-labeled probes or ROX-labeled probes in the Myco. Primer/Probe Mix (see "The sequence of Myco. Positive Control" in Section IX. Appendix).

Proteinase K:
Use this component to remove protein when preparing DNA from culture supernatants or cell suspensions. Proteinase K can be deactivated by incubating at 98℃ for 2 minutes.

III. Storage

-20℃

IV. Materials Required but not Provided

- Real-time PCR amplification instrument and dedicated tubes
  Thermal Cycler Dice™ Real Time System *II* (Cat. #TP900/TP960)＊
  Thermal Cycler Dice Real Time System *Lite* (Cat. #TP700/TP760)＊
  Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific), etc.
  ＊ Not available in all geographic locations. Please check for availability in your area.
- 200 μl, 20 μl, 10 μl micropipettes and tips (with hydrophobic filters)
- Benchtop centrifuge

V. Considerations Before Use

- This kit is designed to detect mycoplasma DNA and can also detect non-viable bacteria. Mycoplasma DNA cannot be detected in some cases, when a mutation or deletion/ insertion occurs within the sequence covered by the Myco Primer/Probe Mix. (Takara Bio is not responsible for any actions taken as a result of analytical determinations made with this product.)
- When a sample is judged as positive, it should be verified also by microbioassay.

VI. Precautions

1) Operate real-time PCR instruments in accordance with the manufacturer's instructions.
2) The chimeric probe and primers are susceptible to degradation by nucleases, and if degraded, cannot provide accurate detection. Take care to avoid nuclease contamination from sources such as perspiration or saliva introduced during sample handling.
3) It is recommended to designate and physically segregate the 3 areas described below for the processes from preparation of reaction mixtures to detection. Avoid opening/ closing tubes containing amplification products in any of these areas:
   - ○ Area 1: reaction mixture preparation and dispensing
   - ○ Area 2: sample preparation
   - ○ Area 3: addition of samples to reaction mixtures, reaction, and detection

   This kit allows amplification and detection to take place simultaneously in real time. Thus, no electrophoresis or other analytical methods are required after the reaction is complete. Never remove amplification products from tubes, as doing so may introduce contamination.

VII. Protocol

[Overview]

1. Sample preparation
2. Real-time PCR instrument set up
3. Preparation of reaction solution and start of reaction
   Prepare reaction solution.
   ↓
   Dispense reaction solution into reaction tubes and add negative control, positive control, and sample.
   ↓
   Set reaction tube in real-time PCR instrument and start reaction.
   ↓
4. Display results
   The amplification plots are displayed in real time.
   ↓
   Reaction completion
   ↓
   Interpretation

VII-1. Sample preparation (work in Area 2)

Prepare samples for *Mycoplasma* detection by any of the following methods using cell cultures grown for 3 - 6 days. We recommend using a Proteinase K-treated sample of culture supernatant or cell suspension for conventional testing for *Mycoplasma* contamination. For the effect of Proteinase K on detection sensitivity, see Section VIII. Experimental Example.
PCR reaction inhibitors in the sample may prevent accurate *Mycoplasma* detection. To test for the presence of inhibitors, perform a control experiment by adding the Myco. Positive Control (1 μl) to the sample (2.5 μl), then detect ROX signal. If the Myco. Positive Control ROX signal is not detected, re-purify the sample DNA using a DNA extraction kit etc to remove inhibitors or try another sample preparation method.

[Method 1 : Proteinase K treatment]

[For cell culture supernatant]

(1) Collect 25 - 100 μl of culture supernatant in a 0.2 ml tube.
(2) Add 1 μl of Proteinase K for every 100 μl of culture supernatant.
(A ratio of 1 μl of Proteinase K to 25 μl of culture supernatant can also be used)
(3) Incubate at 55℃ for 15 minutes in a thermal cycler.
(4) Incubate at 98℃ for 2 minutes to inactivate the enzyme.

[For cell suspension]

(1) Detach adherant cells with a scraper and transfer cells to a tube.
(2) Vortex well if cell aggregation is observed.
(3) Add 1 μl of Proteinase K for every 100 μl of cell suspension.
(A ratio of 1 μl of Proteinase K to 25 μl of cell suspension can also be used)
(4) Incubate at 55℃ for 15 minutes in a thermal cycler.
(5) Incubate at 98℃ for 2 minutes to inactivate the enzyme.

[Method 2 : Using cell culture supernatant directly]

If conventional culture medium, such as Eagle's Medium, has been used for cell culture, it is possible to use the culture supernatant directly. In this case, the amount of supernatant used can be up to 1/10 of the PCR reaction volume.

[Method 3 : Phenol/chloroform/isoamyl alcohol (PCI) extraction of cell culture supernatant]

With this method, the total sample volume can be used for ethanol precipitation. Therefore, the amount of input DNA in the reaction can be increased, improving sensitivity.

Note : Depending on the cell type and culture medium, PCR inhibitors may also be concentrated in this method and cause inhibition of the reaction. We recommend that the control experiment described above be performed.

(1) Transfer 600 μl of culture supernatant to a 1.5 ml tube.
(2) Add 600 μl of phenol saturated with TE buffer and mix well.
(3) Centrifuge at 15,000*g* for 5 minutes at room temperature.
(4) Transfer 500 μl of upper layer to a new 1.5 ml tube.
(5) Add 550 μl of chloroform: isoamyl alcohol (24 : 1) and mix well.
(6) Centrifuge at 15,000*g* for 5 minutes at room temperature.

(7) Transfer 500 μl of upper layer to a new 1.5 ml tube.
(8) Add 500 μl of chloroform:isoamyl alcohol (24 : 1) and mix well.
(9) Centrifuge at 15,000*g* for 5 minutes at room temperature.
(10) Transfer 400 μl of upper layer to a new 1.5 ml tube.
(11) Add 12 μl of 3 M sodium acetate (pH 5.2). A co-precipitating agent can be added if desired.
(12) Add 1 ml of ethanol, mix well, and let stand at -20℃ for 1 hour.
(13) Centrifuge at 15,000*g* for 10 minutes at 4℃.
(14) Remove supernatant and add 500 μl of 70% ethanol.
(15) Centrifuge at 15,000*g* for 10 minutes at 4℃.
(16) Remove supernatant completely and let pellet dry.
(17) Resuspend the pellet in 30 μl of sterile purified water.

VII-2. Reaction preparation

(1) Prepare the reaction mixture on ice (work in Area 1)
Prepare components other than the template in a volume sufficient for the required number of tubes＊1 plus a few extra. Dispense 22.5 μl of the reaction mixture into reaction tubes and cap loosely. Add 2.5 μl of sterile purified water to one of the tubes as a negative control and cap tightly.

Reagent	Volume (one reaction)	Final conc.
2X CycleavePCR Reaction Mix	12.5 μl	1X
Myco. Primer/Probe Mix (5X conc.)	5 μl	1X
Sample, positive control＊1, or sterile purified water	(2.5 μl)＊2	
RNase Free dH_2O	5 μl	
Total	25 μl	

＊1 Use 1 μl of Myco. Positive Control for the positive control reaction and add 1.5 μl of sterile purified water to become a total of 25 μl.
＊2 The test sample or positive control is added in step (2).

【Precaution】
Because real-time PCR relies on the optical measurement of fluorescence, take care must be taken that the tubes do not become dirty. Wear gloves when handling PCR tubes.

(2) Addition of sample (template) (work in Area 3)
Add sample or positive control to each tube except the negative control and cap tightly. Briefly centrifuge the reaction tubes using a benchtop centrifuge and then set in a real-time PCR instrument.

【Precaution】
After the reaction solutions have been prepared, start the reactions within 1 hour.

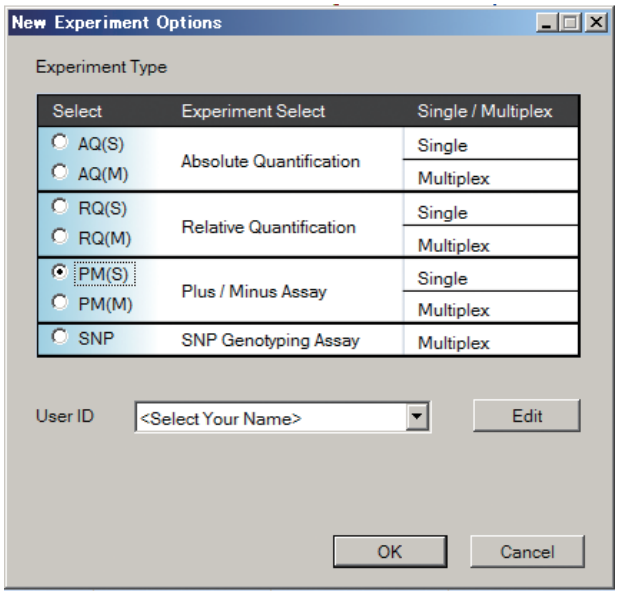
VII-3. Amplification and detection by real-time PCR and interpretation of results (work in Area 3)

Operating procedures differ depending on the real-time PCR instrument used. For specific operating procedures, see the instrument manual. An overview of operation and analysis is described here for the Thermal Cycler Dice Real Time System *II*.

[Thermal Cycler Dice Real Time System *II*]

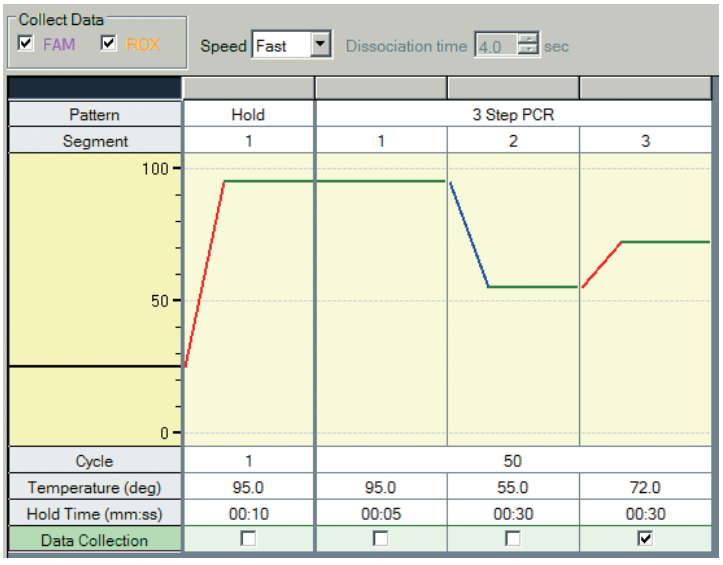
(Thermal Cycler Dice Real Time System Software)

1) Open a new run file. On the "New Experiment Options" screen, select Experiment Type: "Plus/Minus Assay", "Single".

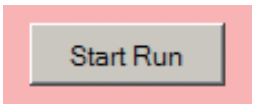


2) On the Thermal Profile Setup screen, make sure both detection filters, FAM and ROX, are checked and the PCR conditions are set as follows.

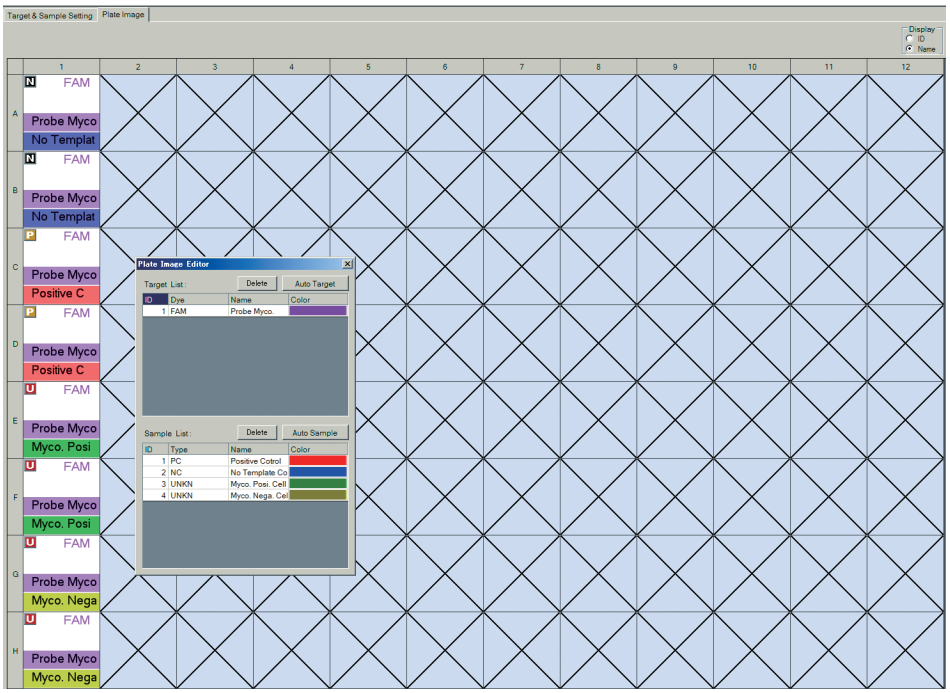
Initial denaturation (Hold)
Cycle : 1
95℃ 10 sec
3 step PCR
Cycle : 50
95℃ 5 sec
55℃ 30 sec
72℃ 30 sec (detection)



3) Click the "Start Run" button on the bottom right-hand corner of the screen to start the reactions.

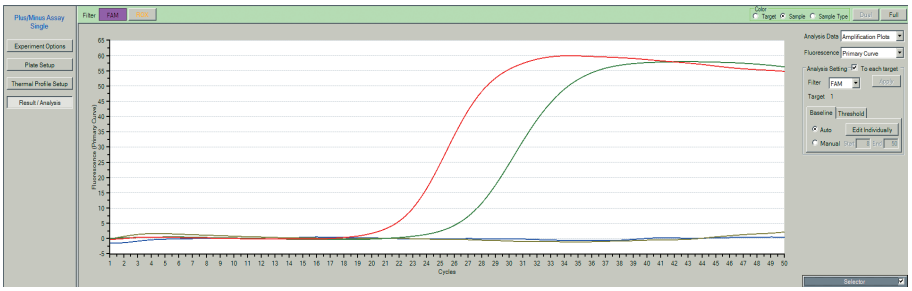


4) Set each well information on the "Plate Setup" screen. Set "Omit" for wells not used in the reaction.



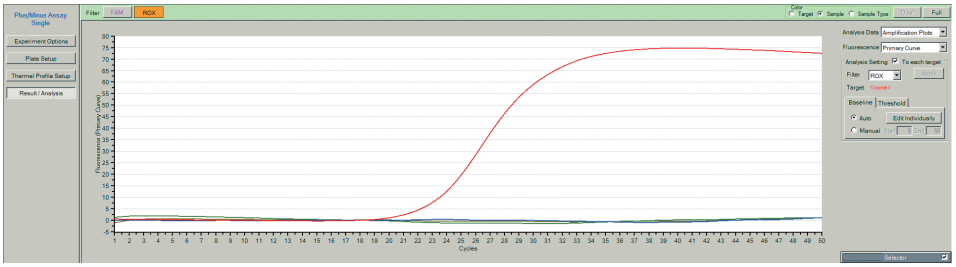
5) After the end of the reaction, check the results on the "Result/Analysis" screen. Select the FAM filter and display amplification plots.

Positive control
Negative control
Sample (Posi)
Sample (Nega)



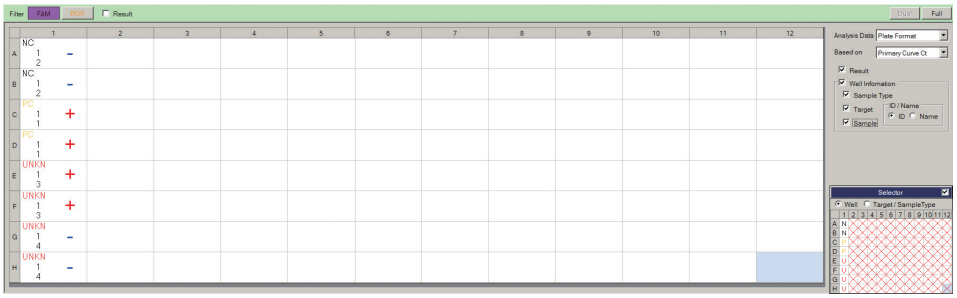
Check the positive control and negative control amplification curves. Make sure that an increase in signal is present for the positive control well (red) and that no signal is detected for the negative control well (navy).

Select ROX in filter and display amplification plots.



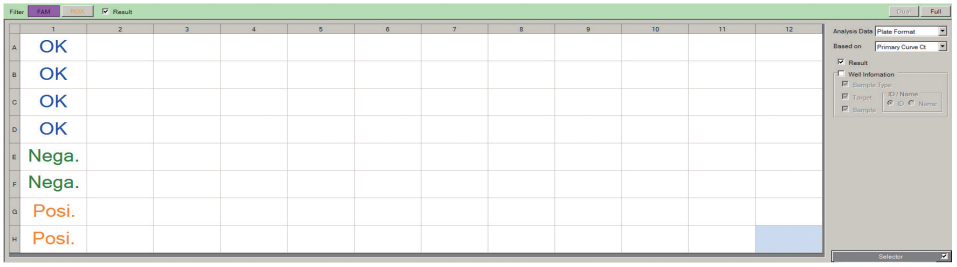
Check the positive control and negative control amplification plots. Make sure that an increase in signal is present for the positive control well (red) and that no signal is detected for the negative control (navy).
In addition, make sure that there is no amplification in the sample wells.

6) To analyze the test samples, select "Plate Format" from the "Analysis data" pull-down menu.



If the "Result" box is not checked:

In the bottom right Selector, results are displayed as negative control (N), positive control (P), and sample (U). In the well display, negative control is displayed as (NC), positive control is displayed as (PC), and sample is displayed as (UNKN). A "+" is displayed for wells in which the target gene was detected and "-" is displayed for wells in which the target gene was not detected.



If the "Result" box is checked:

OK : Control reaction is normal (reaction system has proceeded normally)
OUT : Control reaction is abnormal (reaction system has not proceeded normally)

In sampe wells (U)

Posi : Positive for detection of target gene＊
Nega : Target gene is below limit of detection＊
ND : No interpretation is possible (PCR reaction did not proceed properly)

＊ Based on whether or not threshold values have been exceeded.

[For the Applied Biosystems 7500 Fast Real-Time PCR System]

The same PCR conditions can be used for the Applied Biosystems 7500 Fast Real-Time PCR System as are described for the Thermal Cycler Dice Real Time System *II*.

Select "Quantification-Standard" in "Experiment Properties", then select "TaqMan Reagents" or "Other". (If "Other" is selected, deselect the check mark for "Include Melt Curve"). For "Define Target" in "Plate Setup", set "Reporter" to "FAM" and "Quencher" to "NONE". Set "Passive Reference" to "None" in "Plate Layout".

VIII. Experimental Example

Method:

Renal cell carcinoma cells contaminated with *Mycoplasma* were cultured in a T25 flask. Three days after inoculation, an aliquot of culture supernatant was collected. In addition, the adherent cells were removed from the flask by scraping, and the resulting cell suspension was collected.
25 μl of the culture supernatant and cell suspension were each added to 0.2 ml tubes. For Proteinase K treatment, 1 μl of Proteinase K was added, the mixture was incubated at 55℃ for 15 minutes using a thermal cycler, and then incubated at 98℃ for 2 minutes. 2.5 μl of culture supernatant, cell suspension, and their respective Proteinase K-treated samples were used as templates for real-time PCR. Detection was performed according to the protocol described above.

Results:

When culture supernatant was used, detection was possible in either the Proteinase K-treated sample or the untreated sample. However, the increase in signal in the Proteinase K-treated sample was faster than the untreated sample. Based on the Ct value, sensitivity was improved by a factor of approximately 11 by Proteinase K treatment. When cell suspension was used, detection was not possible for the untreated sample, but amplification was possible in the Proteinase K-treated sample. Moreover, greatest detection sensitivity was achieved using the Proteinase K-treated cell suspension.

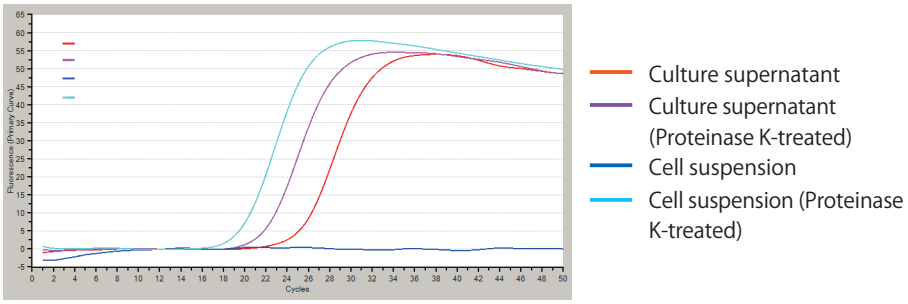


Figure 2. Differences in detection using various samples and treatments.

IX. Appendix

The sequence of Myco. Positive Control

Myco. Positive Control included in this kit has the following sequence inserted at the multiple cloning site of the pMD19 (Simple) vector. The red letters and arrows show the primer position and direction, and the bold capital letters show the cycling probe recognition site. The blue letters indicate RNA.

aaaatgggggtgcggaacattagttagttggtagggtaatggcctaccaagacgatgatgtttagccg

ggccgagaggctgtacggccacactgggactgagatacggcccagactcctacgggaggcagcagtaa

ggaattttccacaatgagcgaaagcttgatggagcgacacagcgtgcaggatgaagttcttcggaatg

taaactgctgttataagggaaggcggccgc taagggctttcaAAGACTGGTTCCggtccttattagaa
Positive Control recognition sequence (*1)

agcgacggcaaactatgtgccagcagcccgcGGTAATACATAGGtcgcagcgttattccggaattatt
Mycoplasma recognition sequence (*2)

gggcgtaaagcgtcctaggtttttgctaaagtctggagttaaatgctgaagctcaacttcagtccgct

ttggatactggcaaaatagaattataaagaggttagcggaattcctagtgaagcggtgggaatgcgta

gatattaggaaggacaccaatagggcgaaggcagctaactggttatatattgacactaagggacgaaa

gcgtggggagcaaacaggattagatacccctggtaggccacgccgtaaacgatgatcat

*1 : Detected with ROX-labeled probe
*2 : Detected with FAM-labeled probe

Primer and probe sequences

Table 1 lists the sequences of the primers and probes in the Myco. Primer/Probe Mix included in this kit. The 16S rRNA sequence for *Mycoplasma* spp., *Bacillus subtilis*, *Escherichia coli*, and several bacterial flora species are also shown below.
The red letters indicate mismatch between the primer or probe sequence and the bacterial genomic sequence, and the gray, blue, and green letters show identical nucleotides.

Table 1. Primer and probe sequences and the genomic DNA sequences for various bacteria

	Foward primer (mix)	Probe	Reverse primer
	TGCGGAACATTAGTTAGTTGG TGCGGCATATCAGCTAGTTGG	GGTAATACaTAGG	TTTACGGCGTGGACTACCAGGG
M. arginini	TGCGGAACATTAGTTAGTTGG	GGTAATACATAGG	TTTACGGCGTGGACTACCAGGG
M. hominis	TGCGGAACATTAGTTAGTTGG	GGTAATACATAGG	TTTACGGCGTGGACTACCAGGG
M. hyorhinis	TGCGGAACATTAGTTAGTTGG	GGTAATACATAGG	TTTACGGCGTGGACTACCAGGG
M. orale	TGCGGAACATTAGCTAGTTGG	GGTAATACATAGG	TTTACGGCGTGGACTACCAGGG
M. salivarium	TGCGGAACATTAGCTAGTTGG	GGTAATACATAGG	TTTACGGCGTGGACTACCAGGG
M. fermentas	TGCGTAACATTAGCTAGTTGG	GGTAATACATAGG	TTTACGGCGTGGACTACCAGGG
M. bovis	TGCGCAACATTAGCTAGTTGG	GGTAATACATAGG	TTTAGGGCGTGGACTACCAGGG
M. arthritidis	TGCGGAACATTAGCTTGTTGG	GGTAATACATAGG	TTTACGGCGTGGACTACCAGGG
M. pirum	TGCGGCATATCAGCTAGTTGG	GGTAATACATAGG	TTTACGGTGTGGACTACTAGGG
M. pneumoniae	TGCGCCATATCAGCTAGTTGG	GGTAATACATAGG	TTTACGGTGTGGACTACTAGGG
A. laidlawii	TGCGGCGCATTAGTTAGTTGG	GGTAATACATAGG	TTTACGGCGTGGACTACCAGGG
B. subtilis	CGCGGCGCATTAGTTAGTTGG	GGTAATACGTAGG	TTTACGGCGTGGACTACCAGGG
E. coli	CAGATGGGATTAGCTAGTAGG	GGTAATACGGAGG	TTTACGGCGTGGACTACCAGGG
Lactobacillus delbrueckii	CGCGGCGCATTAGCTAGTTGG	GGTAATACGTAGG	TTTACGGCATGGACTACCAGGG
Propionibacterium acnes	TCGCGGCTTATCAGCTTGTTGG	GGTGATACGTAGG	TTTACAGCGTGGACTACCAGGG
Actinomyces naeslundii	TCGCGGCCTATCAGCTTGTTGG	GGTAATACGTAGG	TTTACGGCGTGGACTACCAGGG
Staphylococcus aureus	CGCGCTGCATTAGCTAGTTGG	GGTAATACGTAGG	TTTACGGCGTGGACTACCAGGG
Clostridium difficile	CGCGTCTGATTAGCTAGTTGG	GGTAATACGTAGG	TTTACAGCGTGGACTACCAGGG

X. Related Products

TaKaRa PCR Mycoplasma Detection Set (Cat. #6601)*
Thermal Cycler Dice™ Real Time System *II* (Cat. #TP900/TP960)*
Thermal Cycler Dice™ Real Time System *Lite* (Cat. #TP700/TP760)*
0.2 ml 8-strip tube, individual Flat Caps (Cat. #NJ600)
96 well Hi-Plate for Real Time (Cat. #NJ400)
Sealing Film for Real Time (Cat. #NJ500)
48 well snap plate (Cat. #NJ700)
Flat cap for snap plate (Cat. #NJ720)
Plate Sealing Pads (Cat. #9090)

* Not available in all geographic locations. Please check for availability in your area.