Cat. # RR277A

For Research Use

TaKaRa

Mycoplasma qPCR Detection Kit

Product Manual

v201802Da

Table of Contents

I. Description

The Mycoplasma qPCR Detection Kit is designed for detecting mycoplasma DNA by real-time PCR using DNA extracted from a cell suspension. In order to detect a wide range of species, the primer and probe are designed to target the region containing the 16S rRNA and 23S rRNA genes of mycoplasma. Successful DNA extraction from cell samples can be ensured by using the Spike-in Control DNA included in the kit.

This product was developed in a joint research project between Dr. Norio Shimizu and Takara Bio Inc., based on the research results generated by Dr. Shimizu at the Center for Stem Cell and Regenerative Medicine, Tokyo Medical and Dental University, with public support from the Japan Science and Technology Agency (JST) and the Research Center Network for Realization of Regenerative Medicine in the Japan Agency for Medical Research and Development (AMED).

II. Contents (96 Tests): 192 reactions for mycoplasma detection, 96 reactions for Spike-in Control DNA detection*1

1	1. Probe qPCR Mix	2.5X	960 µl x 3
2	2. Primer/Probe Mix (Myco)*2	12.5X	384 µl
3	3. Primer/Probe Mix (Spike-in)*2	12.5X	192 µl
H_2O	4. H_2O		1 ml x 2
5	5. Positive Control (Myco)	(1 x 10^4 copies/µl)	300 µl
6	6. PC Dilution Buffer*3		1 ml x 2
7	7. Spike-in Control DNA		1 ml

*1 Supplementary information on the reaction number:

For each sample, two reactions for mycoplasma detection and one reaction for Spike-in Control DNA detection can be performed in parallel.

# samples analyzed	# mycoplasma detection reactions	# Spike-in Control DNA detection reactions	Total # of reactions
1	2	1	3
7	14	7	21
31	62	31	93

The positive control and negative control reactions below can be performed with every experiment.

	# mycoplasma detection reactions	# Spike-in Control DNA detection reactions	Total # of reactions
Negative control (H_2O)	1	1	2
Positive control	1	0	1

The number of experiments that can be performed with this kit is as follows:
1 sample/experiment: 48 experiments, (4 Myco x 48, 2 Spike-in x 48)
7 samples/experiment: 12 experiments, (16 Myco x 12, 8 Spike-in x 12)
31 samples/experiment: 3 experiments, (64 Myco x 3, 32 Spike-in x 3)

*2 Store protected from light, since it contains a fluorescently-labeled probe.

*3 Use for dilution of the Positive Control (Myco).

III. Storage

-20℃

IV. Materials Required but not Provided

- Nucleic acid extraction kit: NucleoSpin Virus (Cat. #740983.10/.50/.250) etc.
- Real-time PCR instrument and tubes
  Thermal Cycler Dice™ Real-Time System III (Cat. #TP950/TP970/TP980)*
  Thermal Cycler Dice Real-Time System // (Cat. #TP900/TP960)*
  Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific)
  LightCycler 480 system (Roche Diagnostics)
  CFX96 Real-Time PCR Detection System (Bio-Rad) etc.
  * Not available in all geographic locations. Please check for availability in your region.
- Heat block
- High-speed microcentrifuge
- Micropipette and tips

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 Primer/Probe Mix (Myco). (Takara Bio is not responsible for any actions taken as a result of analytical determinations made with this product.)

VI. Precautions

1. Operate real-time PCR amplification instruments in accordance with the manufacturer's instructions.

2. The probe and primers are susceptible to degradation by nuclease and, if degraded, cannot provide accurate detection. Take care to avoid nuclease contamination from perspiration or saliva introduced during sample handling.

3. It is recommended to designate and physically segregate 3 areas as described below for reaction mixture preparation through detection. Avoid opening/closing tubes containing amplification products in any of these areas.

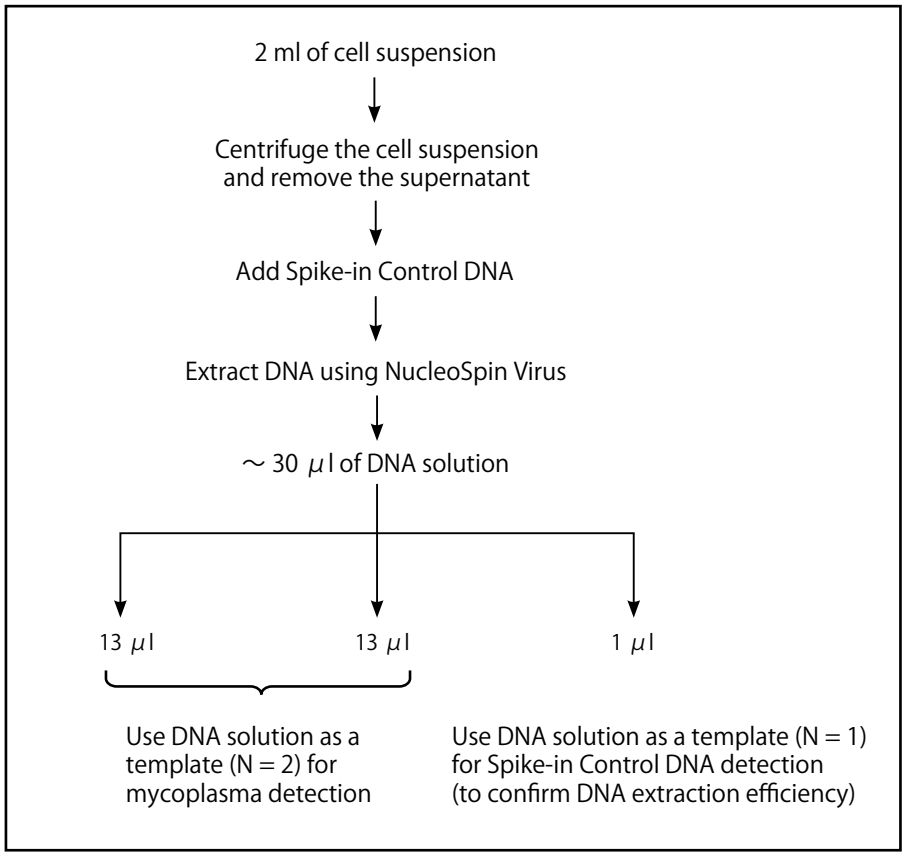
   ○ Area 1: reaction mixture preparation and dispensing
   ○ Area 2: sample preparation
   ○ Area 3: addition of samples to reaction mixtures, reaction, and detection

   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.

4. Results obtained with this kit are interpreted based on analyses by a real-time PCR instrument. Failure of any of the auto functions on the real-time PCR instrument may lead to erroneous interpretation of results. Adjust the settings on the real-time PCR instrument in accordance with the instrument manual when necessary.

VII. Protocol

DNA is extracted from a cell pellet after centrifuging a 2-ml suspension of cultured cells and used as a template for real-time PCR. Two sets of reactions are performed for mycoplasma detection and one set of reactions is performed for Spike-in Control DNA detection.



VII-1. DNA extraction (work in Area 2)

Extract DNA from a sample. In order to monitor the efficiency of the DNA extraction, Spike-in Control DNA is added during the extraction process. As an example, a protocol using NucleoSpin Virus (Cat. #740983.10/.50/.250) is shown below.

1. Centrifuge 2 ml of cell suspension (approximately 2 x 10^6 cells) at 15,000 rpm for 5 minutes and remove 1,810 μl of supernatant (leave 190 μl).
2. Add 10 μl of Spike-in Control DNA.
3. Dissolve the sample as follows:
   Add 5 μl of Liquid Proteinase K (from the NucleoSpin Virus kit).
   Add 200 μl of Buffer VL and vortex for 10 to 15 seconds.
   Add 5.6 μl of Carrier RNA Stock solution*1 and mix gently.
   Let it sit at room temperature for 3 minutes.
4. Add 200 μl of ethanol (96 - 100%) and vortex for 10 to 15 seconds.
   Let it sit at room temperature for 5 minutes.
5. Insert a NucleoSpin Virus Column into a Collection Tube (2 ml).
   Add the solution prepared in Step 4 to the column and centrifuge at 4,000*g* for 3 minutes.*2
   After draining the filtrate, insert the column into a new Collection Tube (2 ml).
6. Wash the column as follows:

   Add 400 μl of Buffer VW1 to the column and centrifuge at 11,000*g* for 30 seconds
   After draining the filtrate, insert the column into a new Collection Tube (2 ml).

   Add 400 μl of Buffer VW2*3 to the column and centrifuge at 11,000*g* for 30 seconds.
   After draining the filtrate, insert the column into a new Collection Tube (2 ml).

   Add 200 μl of Buffer VW2 to the column and centrifuge at the maximum speed (maximum 20,000*g*) for 5 minutes. After draining the filtrate, insert the column into a new Collection Tube (1.5 ml).
7. Dry the column membrane as follows:
   Open the cap of the column and let it dry at 56℃ for 5 minutes.
8. Add 34 μl of RNase-free H_2O preheated at 70℃ and incubate it at room temperature for 3 minutes.
   Centrifuge at 20,000*g* for 3 minutes to elute the DNA.
   If the eluted DNA solution (approximately 30 μl) is not used immediately, store it at -20℃.

*1 Preparation of Carrier RNA stock solution
[when using Cat. #740983.10]
Dissolve 300 μg of freeze-dried Carrier RNA in 300 μl of RNase-free water. Store it at -20℃ after it is dissolved.

*2 If all of the solution did not pass through the column in this centrifugation, perform a high-speed centrifugation (15,000 to 20,800*g*) for 1 minute. If the solution still remains in the upper part of the column, perform the DNA extraction again with a new sample.

*3 Preparation of Buffer VW2
[when using Cat. #740983.10]
Add 24 ml of ethanol (96 to 100%) to 6 ml of Wash Buffer VW2 (Concentrate).

VII-2. Preparation of the real-time PCR reaction mixture

Two types of reaction mixtures are prepared for mycoplasma detection and Spike-in Control DNA detection.

1. Prepare the following reaction mixture on ice. (Work in Area 1.)
   Prepare a sufficient volume of reaction mixture for the required number of reactions plus a few extra.

   < For mycoplasma detection>
   * Prepare a sufficient volume of reaction mixture for the number of samples x 2 + 2 (Positive and Negative Controls).

   [For 1 reaction]

	Reagents	Amount to use
●	Probe qPCR Mix	10 µl
②	Primer/Probe Mix (Myco)	2 µl
	Total	12 µl

   <For Spike-in Control DNA detection>
   * Prepare a sufficient volume of reaction mixture for the number of samples + 1 (Negative Control).

   [For 1 reaction]

	Reagents	Amount to use
●	Probe qPCR Mix	10 µl
③	Primer/Probe Mix (Spike-in)	2 µl
H_2O	H_2O	12 µl
	Total	24 µl

2. Add the reaction mixture prepared in Step 1 to the real-time PCR tubes. (Work in Area 1.)

   For mycoplasma detection : 12 µl per tube
   For Spike-in Control DNA detection : 24 µl per tube

3. Add the DNA solution from Step VII-1-8 (or Positive Control (Myco) or H_2O). (Work in Area 3.)

   For mycoplasma detection : 13 µl per tube
   For Spike-in Control DNA detection : 1 µl per tube

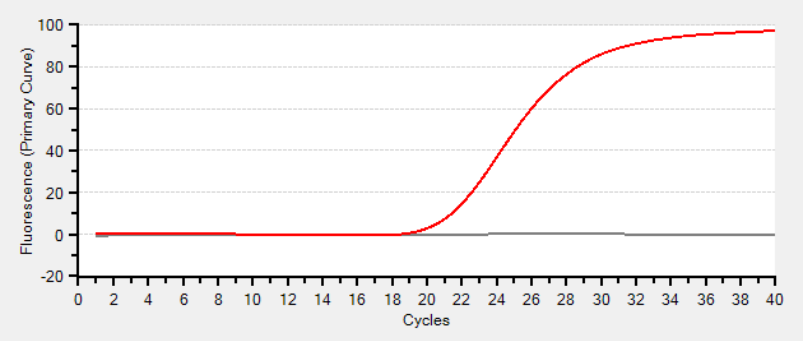
4. Perform the reaction under the following conditions.

   Initial denaturation
   95℃ 30 seconds
   PCR: 5 cycles
   95℃ 5 seconds
   60℃ 1 minute
   PCR: 40 cycles
   90℃ 1 second
   60℃ 1 minute (FAM detection)

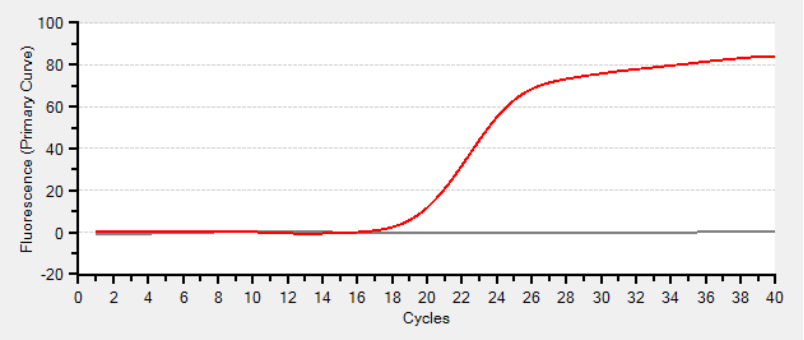
VIII. Experimental Examples

Reaction examples using a Thermal Cycler Dice Real-Time System III are shown below.

<Detection of a Positive Control (Myco)>



<Detection of a Spike-in Control DNA>



IX. Evaluation Analysis

[Precautions regarding the results in this section]
The system does not detect fluorescence in the first 5 PCR cycles, shown in VII-2-4, but does so in the subsequent 40 cycles, shown in VII-2-4. The Ct value indicated on a PCR instrument is obtained as a number which is smaller by 5 than the actual number of PCR cycles. In this section, the Ct value is shown as the actual number of PCR cycles by adding 5 to the Ct value obtained in the experiment.

IX-1. Specificity-1

<Method> Detection by real-time PCR was performed using 7 mycoplasma genomic DNAs available commercially. Approximately 10 pg of DNA was used as a template and duplicate detection (N=2) was performed for each sample.

<Results> Table 1 shows the mycoplasma species used and the Ct values obtained from real-time PCR. Excellent detection performance was observed with all 7 species.

Table 1. Mycoplasma species

No.	Species	ATCC No.	Template	Ct1	Ct2	Average
1	*Mycoplasma arthritidis*	19611D	10 pg	27.4	27.4	27.4
2	*Mycoplasma bovis*	25523D	10 pg	24.4	24.5	24.4
3	*Mycoplasma hominis*	23114D	10 pg	23.2	23.2	23.2
4	*Mycoplasma hyopneumoniae*	25934D	10 pg	24.0	24.0	24.0
5	*Mycoplasma pirum*	25960D	10 pg	26.4	26.4	26.4
6	*Mycoplasma synoviae*	qCRM-25204D	10^4 copies	23.5	23.4	23.4
7	*Spiroplasma citri*	27556D-5	10 pg	25.9	26.0	25.9

IX-2. Specificity-2

<Method> Detection by real-time PCR was performed using 13 bacterial genomic DNAs available commercially. Approximately 100 pg of DNA was used as a template and duplicate detection (N=2) was performed for each sample.

<Results> Table 2 shows the bacterial species used and the Ct values obtained from real-time PCR. Cross-reactivity was not detected with any bacterial genomic DNA.

Table 2. Bacteria other than Mycoplasma

No.	Species	NBRC No.	Template	Ct1	Ct2
1	*Bacillus subtilis*	13719G	100 pg	--	--
2	*Brevibacillus brevis*	100599G	100 pg	--	--
3	*Clostridium acetobutylicum*	13948G	100 pg	--	--
4	*Clostridium kluyveri*	12016G	100 pg	--	--
5	*Escherichia coli*	12713G	100 pg	--	--
6	*Klebsiella pneumoniae*	14940G	100 pg	--	--
7	*Pseudomonas aeruginosa*	106052G	100 pg	--	--
8	*Salmonella enterica subsp. enterica*	13245G	100 pg	--	--
9	*Staphylococcus aureus*	100910G	100 pg	--	--
10	*Streptococcus mutans*	13955G	100 pg	--	--
11	*Streptomyces avermitilis*	14893G	100 pg	--	--
12	*Rhodococcus erythropolis*	100887G	100 pg	--	--
13	*Tetragenococcus halophilus*	12172G	100 pg	--	--

IX-3. Sensitivity

<Method> The cut-off value for obtaining a 95% positive result using this kit was determined using mycoplasma standards for 7 different species (Table 3) from ATCC. 1, 10, and 100 cfu/ml of the mycoplasma standards were added to 10^6 cells/ml of CHO cells. (0.1, 1, and 10 ccu/ml for *M. pneumonia*). DNA from CHO cells containing added mycoplasma was extracted and analyzed using this kit. The mycoplasma standards were diluted stepwise in 4 sets of dilutions. DNA extraction and real-time PCR were repeated 6 times for each 10 cfu/ml mycoplasma addition, providing results for a total of 24 data points. For the 1 cfu/ml and 100 cfu/ml mycoplasma additions, DNA extraction and real-time PCR were repeated twice, providing results for a total of 8 data points.

Table 3. Mycoplasma standards for different species

Species	ATCC No.	Lot No.	Post-preservation titer	Genome copy (GC)
Mycoplasma arginini	23838-TTR	60224014	3.70×10^9 cfu/ml	8.93×10^9 GC/ml
Mycoplasma fermentans	19989-TTR	60316337	1.00×10^9 cfu/ml	8.61×10^9 GC/ml
Mycoplasma salivarium	23064-TTR	60171952	1.67×10^9 cfu/ml	3.80×10^9 GC/ml
Mycoplasma orale	23714-TTR	61060921	3.08×10^8 cfu/ml	1.54×10^9 GC/ml
Mycoplasma hyorhinis	17981-TTR	63478133	8.77×10^8 cfu/ml	1.23×10^9 GC/ml
Acholeplasma laidlawii	23206-TTR	60171953	7.10×10^8 cfu/ml	5.86×10^9 GC/ml
Mycoplasma pneumoniae	15531-TTR	60171955	1.00×10^8 ccu/ml	5.82×10^9 GC/ml

<Results> The average and standard deviation of Ct values for each test are presented in Table 4. The number and percentage of results judged as positive * are shown in Table 5.
The cut-off values showing a 95% positive result are 10 cfu/ml or less for all 7 species of mycoplasma (1 ccu/ml for *M. pneumoniae*).

* Test results showing Ct values of one or two reactions in duplicate are judged as "Positive".

Table 4. Ct values

	Average			SD		
	100 cfu/ml	10 cfu/ml	1 cfu/ml	100 cfu/ml	10 cfu/ml	1 cfu/ml
M. arginini	33.0	36.2	39.7	0.356	0.995	2.003
M. fermentans	31.9	35.5	38.8	0.385	0.828	0.952
M. salivarium	33.3	37.0	39.2	0.532	0.857	1.853
M. orale	33.8	38.1	39.1	0.293	1.336	0.650
M. hyorhinis	31.3	35.4	39.7	0.402	0.550	2.470
A. laidlawii	30.6	35.2	39.2	0.512	1.908	1.585
	10 ccu/ml	1 ccu/ml	0.1 ccu/ml	10 ccu/ml	1 ccu/ml	0.1 ccu/ml
M. pneumoniae	33.2	36.5	40.0	0.397	1.132	1.898

Table 5. Number and percentage of positive test results

	Positive No.			Positive %		
	100 cfu/ml	10 cfu/ml	1 cfu/ml	100 cfu/ml	10 cfu/ml	1 cfu/ml
M. arginini	8/8	24/24	6/8	100.0	100.0	75.0
M. fermentans	8/8	24/24	5/8	100.0	100.0	62.5
M. salivarium	8/8	24/24	6/8	100.0	100.0	75.0
M. orale	8/8	24/24	5/8	100.0	100.0	62.5
M. hyorhinis	8/8	24/24	8/8	100.0	100.0	100.0
A. laidlawii	8/8	24/24	8/8	100.0	100.0	100.0
	10 ccu/ml	1 ccu/ml	0.1 ccu/ml	10 ccu/ml	1 ccu/ml	0.1 ccu/ml
M. pneumoniae	8/8	24/24	3/8	100.0	100.0	37.5

X. Related Products

TaKaRa Mycoplasma qPCR Detection Kit (For Takara Bio instruments) (Cat. #RR278A)*
Virus Test Kit (HIV, HTLV, HCV, HBV, ParvoB19) Ver.2 (Cat. #RR273A)*
Virus Test Kit (EBV, CMV, WNV) Ver.2 (Cat. #RR274A)*
Thermal Cycler Dice™ Real-Time System III with PC (Cat. #TP970)*
Thermal Cycler Dice™ Real-Time System *II* (Cat. #TP900/TP960)*
Thermal Cycler Dice™ Real-Time System *Lite* (Cat. #TP700/TP760)*
0.1 ml 8-strip tube & cap Set (Cat. #NJ903)*
HardFrame Dice™ 0.1ml 96 well qPCR plate (Cat. #NJ904)*
Sealing Film for Real-Time (Adhesive) (Cat. #NJ501)*
0.2 ml 8-strip tube, individual Flat Caps (Cat. #NJ600)*

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