February 27, 2017

Myco Finder Validation Data

Shimadzu Diagnostics Corporation

Research Department

1. Overview

To evaluate the performance of the mycoplasma gene detection kit (Myco Finder), validation data was obtained with reference to the following two information.

1. The Japanese Pharmacopoeia,17th Edition (Ministry of Health, Labour and Welfare Notification No. 64, March 7, 2016)

   Reference Information

   Mycoplasma negative test for cell substrates used in the manufacture of biotechnology-applied pharmaceuticals/biologically-derived pharmaceuticals p 2395 -2399

2. PHARM TECH JAPAN (2016) vol. 32, No. 1, 93 -94

2. Details of Myco Finder Validation and Experimental Conditions

The experimental conditions performed with the real-time PCR devices are shown in Table 1, and the mycoplasma information of this validation is shown in Table 2.

Table 1. Myco Finder validation detection criteria

DNA extraction reagent	QIAGEN: QIAamp UCP DNA Micro Kit
PCR device	BioRad: CFX 96
PCR conditions	95 °C, 10 seconds 98℃, 3 seconds 60℃, 1 second } 45 cycles Fluorescence detection wavelength: FAM + ROX (or FAM + HEX)
Judgment of measurement results	Analysis performed using the software provided with the PCR device (CFX ManagerTM software): Regression method, Baseline Subtracted Curve Fit mode

Table 2. Mycoplasma reference material used for validation

For validation, the reference materials prepared under Associate Professor Norio Shimizu of the Tokyo Medical and Dental University Research Center for Regenerative Medicine were used.

Species name	GC (copies/mL) *	CFU (CFU/mL)	GC/CFU
A. laidlawii	3.9 x 10^{9}	3.5 x 10^{8}	11.2
M. arginini	4.1 x 10^{9}	2.2 x 10^{9}	1.8
M. fermentans	1.3 x 10^{10}	3.3 x 10^{9}	3.9
M. hyorhinis	1.4 x 10^{9}	4.2 x 10^{8}	3.4
M. orale	1.3 x 10^{10}	8.3 x 10^{8}	16.3
M. pneumoniae	6.0 x 10^{8}	2.9 x 10^{7}	20.7
M. salivarium	4.2 x 10^{9}	2.5 x 10^{9}	1.7

*Copies/mL = [DNA contents (μg/mL)] x 10^{6} x (0.978 x 10^{9})/[genome size]

3. Specificity

3-1. Specific amplification of target region

PCR using primers used in the Myco Finder was performed using DNA extracted from the reference materials of the seven species shown in Table 2 as a template, followed by sequence analysis. The results showed that the primers of Myco Finder could specifically amplify the seven mycoplasma species (Table 3).

Species name	Homology
A. laidlawii	100%
M. arginini	99%*
M. fermentans	99%*
M. hyorhinis	99%*
M. orale	99%*
M pneumoniae	100%
M salivarium	99%*

•Include & Undetectable

3-2. Cross-reactivity with other species

Although not included in the validation method specified in the Japanese Pharmacopoeia,17th Edition, the cross-reactivity of the genomic DNA of the 25 bacterial species, eight fungal species, and three mammalian cell species shown in Table 4 was evaluated. Genomic DNA was added to achieve at least 1 ng/reaction in each case. As a result of the evaluation, no cross-reactivity was observed for any of the genomic DNAs listed in Table 4.

Table 4. List of species tested for cross-reactivity

25 bacterial species	8 fungal species	3 mammalian cell species
Bacteroides vulgatus	*Cryptococcus neoformans*	Raji Cell (Human)
Bacillus subtilis	*Candida albicans*	Mouse T lymphocyte (Mouse)
Clostridium acetobutylicum	*Mucor circinelloides*	CHO cell (Chinese hamster)
Clostridium kluyveri	*Cunninghamella echinulata*	
Clostridium sporogenes	*Rhizomucor pusillus*	
Escherichia coli	*Absidia corynbifera*	
Enterococcus faecalis	*Scedosporium proficans*	
Gluconacetobacter xylinus	*Pneumocystis carinii*	
Klebsiella pneumoniae		
Lactobacillus acidophilus		
Lactobacillus bulgaricus		
Lactobacillus casei		
Propionibacterium acnes		
Salmonella enterica		
Staphylococcus aureus		
Staphylococcus epidermidis		
Streptococcus mutans		
Streptococcus pneumoniae		
Streptococcus bovis		
Streptococcus avermitilis		
Rothia dentocariosa		
Tetragenococcus halophilus		
Kocuria rhizophila		
Pseudomonas aeruginosa		

4. Detection Sensitivity

For the seven bacterial species to be detected shown in Table 2, eight different dilution series of 1, 10, and 100 CFU/mL were prepared, and a cell suspension of CHO cell DG 44 strain $1X10^6$ cells/mL was added to each of them to serve as a sensitivity test sample. These were used for performing three tests on separate days, for 24 tests in total. Only the above cell suspensions ($1X10^6$ cells/mL) were used as negative samples.

The results of the detection sensitivity test are shown in Tables 5 to 11. For all species, 10 CFU/mL was detectable with a probability of more than 95%. This indicates that Myco Finder has the sensitivity to meet the requirement of 10 CFU/mL, as an alternative to culture.

Table 5. Sensitivity test results (*A. laidlawii*)

Spike (CFU/mL)	Run			Total	Positive rate (%)
	1	2	3		
100	8/8	8/8	8/8	24/24	100.0
10	8/8	8/8	8/8	24/24	100.0
1	6/8	7/8	8/8	21/24	87.5

3/3 means detected 3 times out of 3 tests.

Table 6. Sensitivity test results (*M. arginini*)

Spike (CFU/mL)	Run			Total	Positive rate (%)
	1	2	3		
100	8/8	8/8	8/8	24/24	100.0
10	8/8	8/8	8/8	24/24	100.0
1	4/8	5/8	4/8	13/24	54.2

3/3 means detected 3 times out of 3 tests.

Table 7. Sensitivity test results (*M.fermentans)*

Spike (CFU/mL)	Run			Total	Positive rate (%)
	1	2	3		
100	8/8	8/8	8/8	24/24	100.0
10	8/8	8/8	8/8	24/24	100.0
1	3/8	5/8	7/8	15/24	62.5

3/3 means detected 3 times out of 3 tests.

Table 8. Sensitivity test results (*M.hyorhinis)*

Spike (CFU/mL)	Run			Total	Positive rate (%)
	1	2	3		
100	8/8	8/8	8/8	24/24	100.0
10	8/8	8/8	8/8	24/24	100.0
1	1/8	3/8	4/8	8/24	33.3

3/3 means detected 3 times out of 3 tests.

Table 9. Sensitivity test results (*M.orale)*

Spike (CFU/mL)	Run			Total	Positive rate (%)
	1	2	3		
100	8/8	8/8	8/8	24/24	100.0
10	8/8	8/8	8/8	24/24	100.0
1	4/8	4/8	4/8	12/24	50.0

3/3 means detected 3 times out of 3 tests.

Table 10. Sensitivity test results (*M. pneumoniae*)

Spike (CFU/mL)	Run			Total	Positive rate (%)
	1	2	3		
100	8/8	8/8	8/8	24/24	100.0
10	8/8	8/8	8/8	24/24	100.0
1	3/8	2/8	5/8	10/24	41.7

83/3 means detected 3 times out of 3 tests.

Table 11. Sensitivity test results (*M. salivarium)*

Spike (CFU/mL)	Run			Total	Positive rate (%)
	1	2	3		
100	8/8	8/8	8/8	24/24	100.0
10	7/8	8/8	8/8	23/24	95.8
1	5/8	3/8	2/8	10/24	41.7

3/3 means detected 3 times out of 3 tests.

5. Robustness

Regarding the effect of the differences in the detection devices on the performance of Myco Finder, the performance of Myco Finder was evaluated when LightCycler 480 (Roche) and GVP9600 (Shimadzu Corporation) were used as real-time PCR devices.The results showed that using different real-time PCR devices does not affect Myco Finder detection performance.

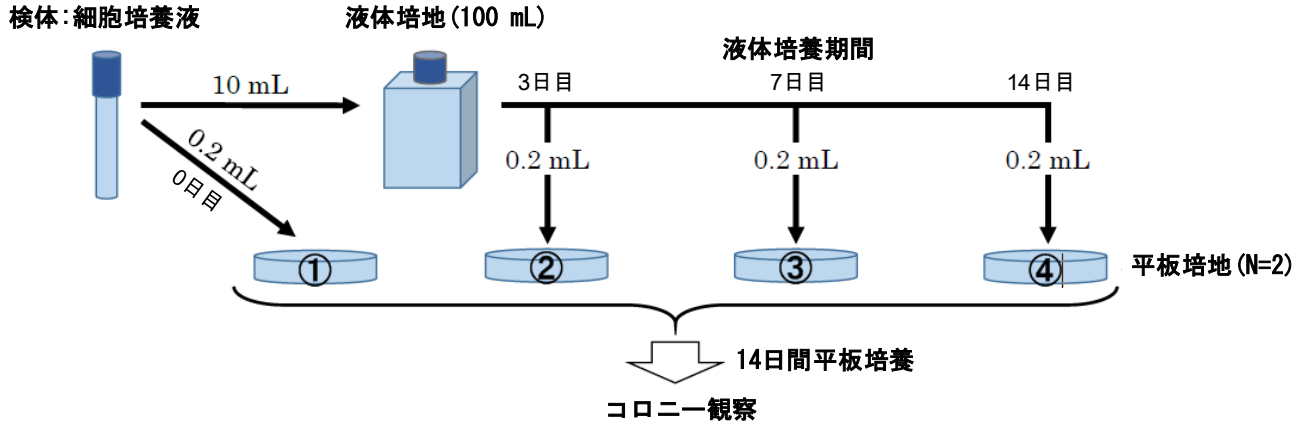
Table 12. Effect of differences in PCR devices on detection performance

Species name	PCR device	Positive count (10 CFU/mL)
A. laidlawii	LightCycler 480	3/3
	GVP 9600	3/3
M. arginini	LightCycler 480	3/3
	GVP 9600	3/3
M. fermentans	LightCycler 480	3/3
	GVP 9600	3/3
M. hyorhinis	LightCycler 480	3/3
	GVP 9600	3/3
M.orale	LightCycler 480	3/3
	GVP 9600	3/3
M. pneumoniae	LightCycler 480	3/3
	GVP 9600	3/3
M. salivarium	LightCycler 480	3/3
	GVP 9600	3/3

3/3 means detected 3 times out of 3 tests.

6. Equivalence Test – Culture Method

In parallel with the detection of 10 CFU/mL of the seven bacterial species to be detected as shown in Table 2, the same sample (10 CFU/mL) was used for detection by the culture method. The conditions as an alternative to the culture method (10 CFU/mL) were met, and the same sample was detected in the culture method.



	Sample: Cell culture medium
	Day 0
	Liquid medium (100 mL)
	Day 3
	Liquid incubation period
	Day 7
	Plate culture for 14 days
	Colony observation
	Day 14
	Plate medium (N = 2)

Figure 1. Overview of culture method

1. Prepare a test sample of 10 CFU/1 mL cell suspension of Mycoplasma Reference Standard for seven mycoplasma species.
2. Inoculate 0.2 mL of each sample in a plate medium, and incubate at 37 °C for 14 days (①).
3. Inoculate 10 mL of each sample in 100 mL of Hayflick (Millipore) liquid medium, and incubate at 37 C. On days 3, 7, and 14 of incubation, extract 0.2 mL from each medium and incubate in a plate medium at 37 °C for 14 days (②④).
4. Check for the presence/absence of mycoplasma colonies at the time of ① to ④.

Table 13. Comparison of culture results with NAT

Species name	No. of days of liquid culture				Judgment	NAT (using Myco Finder)
	① Day 0	② Day 3	③ Day 7	④ Day 14		
A. laidlawii	**Yes**	**Yes**	**Yes**	**Yes**	**+**	**+**
M. arginini	**Yes**	**Yes**	**No**	**No**	**+**	**+**
M. fermentans	**Yes**	**Yes**	**Yes**	**Yes**	**+**	**+**
M. hyorhinis	**Yes**	**Yes**	**Yes**	**Yes**	**+**	**+**
M. orale	**Yes**	**Yes**	**No**	**No**	**+**	**+**
M. pneumoniae	**No**	**No**	**Yes**	**Yes**	**+**	**+**
M. salivarium	**No**	**Yes**	**Yes**	**Yes**	**+**	**+**

Positive **+:**

The presence of one or more colonies in the plate medium during any incubation period was considered positive.

M. arginini, M. orale returned negative results after day 7, presumably as a result of a long liquid incubation period and the death of all bacteria that grew.

End

September 29, 2021

Myco Finder Validation Data

Additional Data

Shimadzu Diagnostics Corporation

Research Department

1. Overview

To evaluate the performance of the mycoplasma gene detection kit (Myco Finder), validation data was obtained with reference to the following two information. New data on robustness were obtained between February 2021 and August 2021 and are reported as additional data.

1. The Japanese Pharmacopoeia,17th Edition (the Ministry of Health, Labour and Welfare Notification No. 64, March 7, 2016) Reference Information
   Mycoplasma negative test for cell substrates used in the manufacture of biotechnology-applied pharmaceuticals/biologically-derived pharmaceuticals p 2395 -2399
2. PHARM TECH JAPAN (2016) vol. 32, No. 1, 93 -94

2. Experimental Conditions

The experimental conditions performed with the real-time PCR devices are shown in Table 1, and the mycoplasma information of this validation is shown in Table 2.

Table 1. Myco Finder validation detection criteria

DNA extraction reagent	QIAGEN CO., LTD.: QIAamp UCP DNA Micro Kit **Kanto Chemical Co., Inc.:** Cica geneusR Total DNA Prep Kit (for tissue)
PCR devices	**Bio-Rad** Laboratories, Inc.: CFX Connect, CFX Opus
PCR conditions	95 °C, 10 seconds 98℃, 3 seconds 60℃, 1 second } 45 cycles Fluorescence detection wavelength: FAM + ROX (or FAM + HEX)
Judgment of measurement results	Analysis performed using the software provided with the PCR device (CFX ManagerTM software): Regression method, Baseline Subtracted Curve Fit mode

Table 2. Mycoplasma reference material used for validation

Reference materials purchased from ATCC were used to obtain additional data.

Species name	Strain	Lot No.	GC (copies/mL) *	CFU (CFU/mL)	GC/CFU
A. laidlawii	ATCC 23206 - TTR	70023819	5.5×10^{9}	1.3×10^{9}	4.2
M. arginini	ATCC 23838 - TTR	70018034	1.0×10^{1010}	1.7×10^{1010}	0.6
M. fermentans	ATCC 19989 - TTR	70015063	7.5×10^{9}	7.0×10^{9}	1.1
M. hyorhinis	ATCC 17981 - TTR	70015024	1.5×10^{9}	1.9×10^{9}	0.8
M. orale	ATCC 23714 - TTR	70027393	1.1×10^{1010}	1.8×10^{1010}	0.6
M. pneumoniae	ATCC 15531 - TTR	70022921	7.0×10^{9}	1.0×10^{9}	7.0
M. salivarium	ATCC 23064 - TTR	70025854	1.2×10^{1010}	8.6×10^{9}	1.4

*Copies/mL = [DNA contents (g/mL)] x 10^{6} x (0.978 x 10^{9})/[genome size]

3. Additional Robustness Data

3-1. Verification of differences in detection devices

Regarding the effect of the differences in the detection devices on the performance of Myco Finder, the performance of Myco Finder was evaluated when CFX Connect and CFX Opus were used as real-time PCR devices.The results showed that using CFX Connect and CFX Opus does not affect Myco Finder detection performance.

Table 3. Effect of differences in PCR devices on detection performance ①

Species name	PCR device	Positive count (10 CFU/mL)
A. laidlawii	CFX Connect	3/3
M. arginini	CFX Connect	3/3
M. fermentans	CFX Connect	3/3
M. hyorhinis	CFX Connect	3/3
M. orale	CFX Connect	3/3
M pneumoniae	CFX Connect	3/3
M. salivarium	CFX Connect	3/3

3/3 means detected 3 times out of 3 tests.

Table 4. Effect of differences in PCR devices on detection performance ①

Species name	PCR device	Positive count (10 CFU/mL)
A. laidlawii	CFX Opus	3/3
Margin	CFX Opus	3/3
M fermentans	CFX Opus	3/3
M. hyorhinis	CFX Opus	3/3
M. orale	CFX Opus	3/3
M pneumoniae	CFX Opus	3/3
M. salivarium	CFX Opus	3/3

3/3 means detected 3 times out of 3 tests.

3-2. Verification of DNA extraction reagent

Regarding the effect of the differences in DNA extraction reagents on the performance of Myco Finder, the performance of Myco Finder was evaluated by using the Cica geneusR Total DNA Prep Kit as a DNA extraction reagent. The results showed that the performance of the Myco Finder was not affected by the use of the Cica geneusR Total DNA Prep Kit.

Table 5. Effect of differences in DNA extraction reagents on detection performance

Species name	Positive count		
	Worker A	Worker B	Worker C
A. laidlawii	3/3	3/3	3/3
M. arginini	3/3	3/3	3/3
M. fermentans	3/3	3/3	3/3
M. hyorhinis	3/3	3/3	3/3
M. orale	3/3	3/3	3/3
M. pneumoniae	3/3	3/3	3/3
M. salivarium	3/3	3/3	3/3

3/3 means detected 3 times out of 3 tests.

End