Mycoplasma gene detection kit **March 31, 2016**

Myco Finder

Validation Data

Shimadzu Diagnostics Corporation

1. Overview

Validation data was obtained in reference to the following information in order to validate the performance of Myco Finder, a mycoplasma gene detection kit.

1. Japanese Pharmacopoeia 17th Edition (March 7, 2016 The MHLW Ministerial Notification No. 64)
   Reference information p2395-2399
   "Negative Tests for Mycoplasma of Cell Substrate for the Production of Medicines Applied with Biotechnology and Medicines Originated in Biology"
2. PHARM TECH JAPAN (2016) vol.32, No.1, 93-94

2. Myco Finder validation items and test conditions

Table 1 shows reagents used for extracting DNAs and conditions of the real-time PCR test, and Table 2 shows information about mycoplasma reference strains used for validation.

Table 1. Myco Finder validation detection condition

DNA extraction reagent	QIAGEN's DNeasy Blood and Tissue Kit
PCR system	BioRad's CFX96
PCR condition	95°C, 10 seconds 98°C, 3 seconds 60°C, 1 second } 45cycles Fluorescence detection wavelength: FAM + ROX (or FAM + HEX)
Judgment of measurement result	Performed using the software that comes with PCR system (CFX Manager™ Software). Analysis method: Regression method, Baseline Subtracted Curve Fit mode

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

Table 2. Mycoplasma reference strains used for validation

Reference strains produced under the supervision of Mr. Norio Shimiu, Assistant Professor of Tokyo Medical and Dental University Center for Stem Cell and Regenerative Medicine were used for validation.

Strain name	GC (copies/mL)*	CFU (CFU/mL)	GC/CFU
M. pneumoniae	6.0E+08	2.9E+07	20.7
M. hyorhinis	1.4E+09	4.2E+08	3.4
A. laidlawii	3.9E+09	3.5E+08	11.2
M. fermentans	1.3E+10	3.3E+09	3.9
M. arginini	4.1E+09	2.2E+09	1.8
M orale	1.3E+10	8.3E+08	16.3
M salivarium	4.2E+09	2.5E+09	1.7

3. Specificity

Using the DNAs extracted from the 7 reference strains listed in the Table 2 as templates, PCR was performed with Myco Finder's primer, and then sequence analysis was performed. The result showed that Myco Finder's primer can specifically amplify the 7 Mycoplasma reference strains (Table 3).

Table 3. Sequence analysis result

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

*The 1% difference derives from the base that was "undetectable (N)" in the sequence analysis.

4. Detection sensitivity

8 sets of serial dilutions for each type of test strain on Table 2 were made to yield 1, 10, and 100 CFU/mL, and a cell suspension of CHO-DG44 cells at density of $1x10^6$ cells/mL was seeded in each bacterial suspension to prepare samples for the sensitivity test. Using these samples, sensitivity test was performed three times (each on different days) for each type of test strain to perform 24 tests in total. The only negative sample was the cell suspension mentioned above ($1x10^6$ cells/mL).

Result of the sensitivity test is shown in Table 4 through Table 10. For all strains, 10 CFU/mL was detectable with a probability of more than 95%. This shows that Myco Finder is able to detect 10 CFU/mL, having a sensitivity level required of an alternative to culture method.

Table 4. Sensitivity Test Result *(M. orale)*

Spike (CFU/mL)	Run 1	Run 2	Run 3	Total
100	8/8*	8/8	8/8	24/24
10	**8/8**	**7/8**	**8/8**	**23/24**
1	8/8	4/8	7/8	19/24

*Number of detections/Number of tests

Table 5. Sensitivity Test Result (*M pneumoniae)*

Spike (CFU/mL)	Run 1	Run 2	Run 3	Total
100	8/8*	8/8	8/8	24/24
10	**8/8**	**8/8**	**8/8**	**24/24**
1	6/8	5/8	6/8	17/24

*Number of detections/Number of tests

Table 6. Sensitivity Test Result (*M arginini)*

Spike (CFU/mL)	Run 1	Run 2	Run 3	Total
100	8/8*	8/8	8/8	24/24
10	**8/8**	**8/8**	**7/8**	**23/24**
1	4/8	6/8	7/8	17/24

*Number of detections/Number of tests

Table 7. Sensitivity Test Result (*M fermentans)*

Spike (CFU/mL)	Run 1	Run 2	Run 3	Total
100	8/8*	8/8	8/8	24/24
10	**8/8**	**8/8**	**7/8**	**23/24**
1	7/8	7/8	4/7	18/24

*Number of detections/Number of tests

Table 8. Sensitivity Test Result *(M. hyorhiniS)*

Spike (CFU/mL)	Run 1	Run 2	Run 3	Total
100	8/8*	8/8	8/8	24/24
10	**8/8**	**8/8**	**8/8**	**24/24**
1	5/8	5/8	3/8	13/24

*Number of detections/Number of tests

Table 9. Sensitivity Test Result *(M. salivarium*

Spike (CFU/mL)	Run 1	Run 2	Run 3	Total
100	8/8*	8/8	8/8	24/24
10	**8/8**	**7/8**	**8/8**	**23/24**
1	6/8	4/8	3/8	13/24

*Number of detections/Number of tests

Table 10. Sensitivity Test Result *(A. laidlawii)*

Spike (CFU/mL)	Run 1	Run 2	Run 3	Total
100	8/8*	8/8	8/8	24/24
10	**8/8**	**7/8**	**8/8**	**23/24**
1	6/8	6/8	8/8	20/24

*Detected number/Number of tests

5. Robustness

Myco Finder's performance was evaluated by increasing/decreasing the amount of samples from the defined amount (25µL). As a result, it was found that detection result was not affected when the amount of sample was within 25µL ±5%.

Table 11. Effect of increased/decreased sample amount to positive ratio

	Percent change*						
Strain name	**-20%**	**-10%**	**-5%**	**0%**	**+5%**	**+10%**	**+20%**
M. pneumoniae	3/3**	3/3	**3/3**	**3/3**	**3/3**	3/3	3/3
M. hyorhinis	0/3	2/3	**3/3**	**3/3**	**3/3**	3/3	2/3
A. laidlawii	3/3	3/3	**3/3**	**3/3**	**3/3**	3/3	3/3
M fermentans	3/3	3/3	**3/3**	**3/3**	**3/3**	2/3	3/3
M. arginini	3/3	3/3	**3/3**	**3/3**	**3/3**	3/3	3/3
M. orale	1/3	2/3	**3/3**	**3/3**	**3/3**	2/3	0/3
M salivarium	1/3	2/3	**3/3**	**3/3**	**3/3**	2/3	0/3

* The amount of sample was increased/decreased from 25µL as a standard.

** Detected number/Number of tests

6. Equivalence test ~Culture method~

In parallel with the detection test of 10 CFU/mL of the 7 strains shown in Table 2, detection test of the same sample (10 CFU/mL) by culture method was performed. As a result, the same sample was also detectable by culture method. This shows that Myco Finder meets the condition (10 CFU/mL) required of an alternative to culture method.

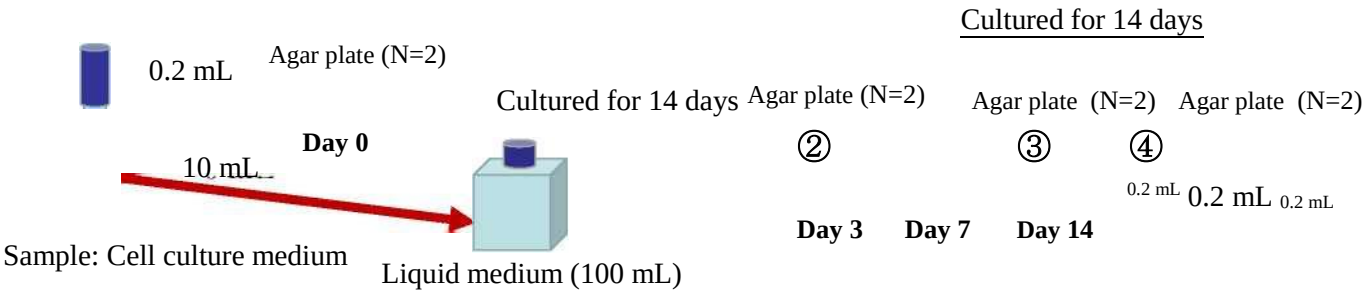

Figure 1. Procedure of culture method

1. For each of the seven Mycoplasma strains, prepare a sample that contains reference strains at 10 CFU/1mL of cell suspension.
2. Inoculate the agar plate with 0.2 ml of each sample, and culture at 37°C for 14 days ((1)).
3. Inoculate 100 mL of Hayflick (Millipore) liquid medium with 10 mL of each sample, and culture at 37°C. Take 0.2 mL of each culture solution on day 3, 7 and 14, and inoculate the agar plate with it, and then culture at 37°C for 14 days ((2)~(4)).
4. Confirm the existence of Mycoplasma colonies at the processes (1) through (4) above.

Table 12 Result of culture method

Strain name	Length of incubation			
	①Day 0	②Day 3	③Day 7	④Day 14
M. pneumoniae	—	—	+	+
M. hyorhinis	+	+	+	+
A. laidlawii	+	+	+	+
M fermentans	+	+	+	+
M. arginini	+	+	—	—
M orale	+	+	—	—
M. salivarium	—	+	+	+

＋:Positive, －:Negative

When more than 1 colonies were confirmed on plate agar within the culture period, we judged it positive.

The result of *M. arginine* and *M. orale* after day 7 were negative, but this is because all the grown strains had died by then because incubation was performed too long.

7. Reference information

~Cross-reactivity~

Although this is not listed in the Japanese Pharmacopoeia 17th Edition as validation method, cross-reactivity to the genomic DNAs of the 25 types of bacteria, 8 types of funguses, and 3 types of mammalian cells listed in the Table 13 was also tested. All genomic DNAs were added to the sample so that concentration will be 1ng/reaction. The result showed that no cross-reaction was observed against any of the genomic DNAs.

Table 13. List of strains for which false-positive reaction was tested

25 types of bacteria	8 types of funguses	3 types of mammalian cells
Bacteroides vulgatus		Raji Cell (human)
Bacillus subtilis	*Cryptococcus neoformans Candida*	Mouse T lymphocyte (mouse)
Clostridium acetobutylicum	*albicans*	CHO cell (hamster)
Clostridium kluyveri	*Mucor circinelloides*	
Clostridium sporogenes	*Cunninghamella echinulata*	
Escherichia coli	*Rhizomucor pusillus Absidia*	
Enterococcus faecalis	*corynbifera Scedosporium*	
Gluconacetobacter xylinus	*prolificans Pneumocystis carinii*	

Klebsiella pneumoniae Lactobacillus acidophilus Lactobacillus bulgaricus Lactobacillus casei Propionibacterium acnes Sallmonella enterica Staphylococcus aureus Staphylococcus epidermidis Streptococcus mutans Streptococcus pneumoniae Streptococcus bovis Streptococcus avermitilis Rhodococcus erythropolis Rothia dentocariosa Tetragenococcus halophilus Kocuria rhizophila Pseudomonas aeruginosa

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For more information including actual values of validation data, please contact sales rep of Shimadzu Diagnostics Corporation or customer support (TEL: 03-5846-5707).