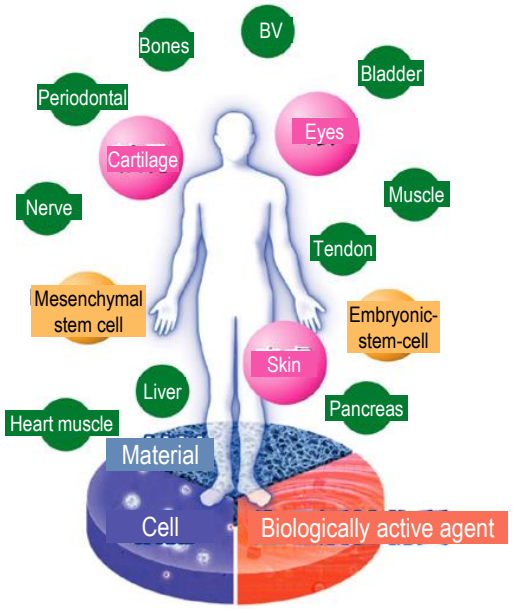
1-1. Why mycoplasma testing is necessary?

Due to the changes in the laws (Act on Securing Quality, Efficacy and Safety of Products Including Pharmaceuticals and Medical Devices, Act on the Safety of Regenerative Medicine Products Safety, etc.) in 2012, commercial application of regenerative medicine products is now allowed. In line with this, mycoplasma testing has become mandatory as part of the tests to secure safety of regenerative medicine products.

<Safety test of regenerative medicine products and bio-pharmaceuticals>

- Sterility Test
- Bacterial Endotoxins Test
- Virus Test
- Mycoplasma Testing



Source: blog.goo.ne.jp/

<Issues related to safety test>

- It is hard to secure sufficient amount of final products of regenerative medicine as test samples because there are limited amount of final products.

- As regenerative medicine products need to be used soon after production, rapid test method is required.

Japanese Pharmacopoeia 17th Edition: Nucleic Acid Amplification Technique was added as a rapid mycoplasma testing method

1-2. What is mycoplasma?

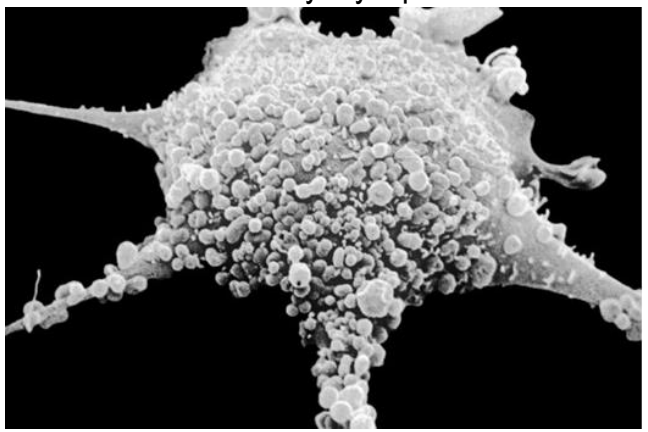
Mycoplasma, Ureaplasma, Spiroplasma, Acholeplasma, and other bacteria that belong to Mollicutes.

<Characteristics>

- **Widely distributed in the animal and plant kingdoms and infection shows a species-specificity.**
- **Infect humans through researchers who perform cell culture or serum used for cell culture, etc.**
- **Many have resistance to antibiotics.**
- **Pass through the filters for sterilization (0.22μm) because of the small size.**
- **Infected cells do not exhibit cytotoxic effect.**

⇒*It is hard to detect mycoplasma contamination*

Fibroblast infected by Mycoplasma



EXCLI J. 2014; 13: 300−322

It is reported that cultured cells in many labs are *often* contaminated by mycoplasma.
⇒Development of medical products using contaminated cells will result in severe consequences.

- Cell culture substrates used for development of medical products
- Cell/tissue-based medical products

} A proper mycoplasma testing has to be performed.

1-4. The 7 types of strains to be detected under Japanese Pharmacopoeia 17th Edition

7 types of strains were selected from the following perspectives as mycoplasma strains that may contaminate cell substrates used for development of medical products in Japan.

- Frequency of contamination and phylogenetic point of view
- Animal-derived components used in cell culture

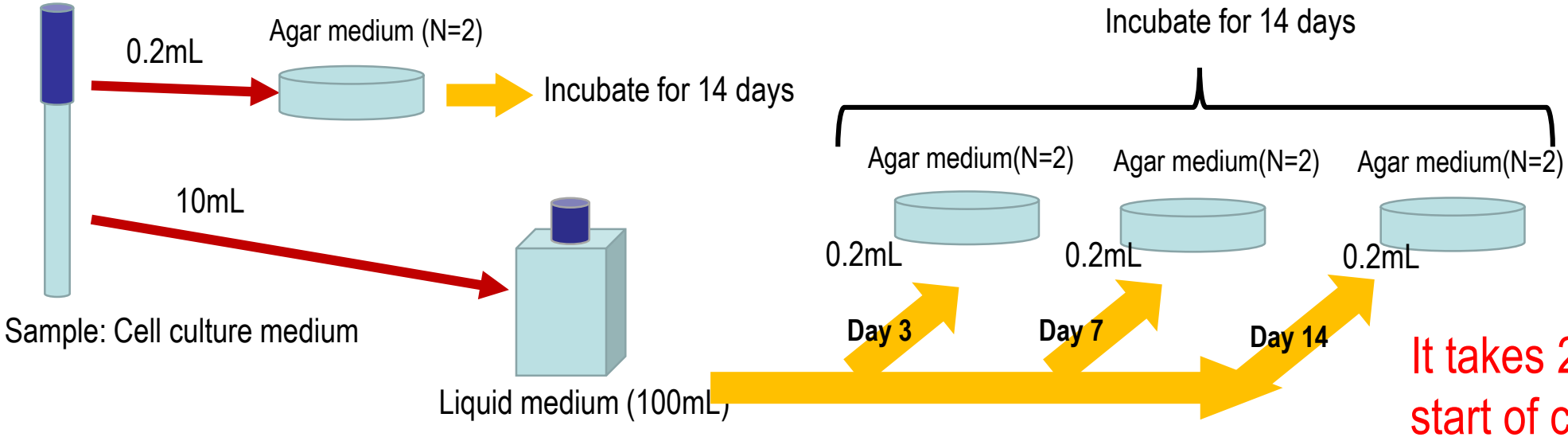
Strain name	Natural host
Mycoplasma hyorhinis	Pig
Mycoplasma orale	Human
Mycoplasma pneumoniae	Human
Mycoplasma salivarium	Human
Acholeplasma laidlawii	Cow
Mycoplasma fermentans	Human
Mycoplasma arginini	Cow / Goat

1-5. Method A: Culture method

<Test method>

Inoculate medium (liquid medium, agar medium) with the sample and culture to detect colonies specific to mycoplasma.

Medium	Amount of sample	No. of tests per sample	Length of incubation
Liquid medium (100mL/medium)	10mL or more	1 or more culture bottles	For 14 days ⇒Take 0.2mL on day 3, 7, and 14, and inoculate agar plates with them (N=2), and then incubate for 14 days.
Agar medium	0.2mL	2 or more agar plates	For 14 days



It takes 28 days from the start of culture to the end of the test

1-6. Method B: DNA staining method

<Test method>

This method indirectly detects mycoplasma that have grown in cell culture by inoculating the indicator cells (Vero cells) with the test sample and staining the mycoplasma DNA with fluorescent staining dye to observe small extra-nuclear fluorescence spots.

Incubate indicator cells (Vero cells) in culture dish for one day
(Use 2 or more dishes per sample)

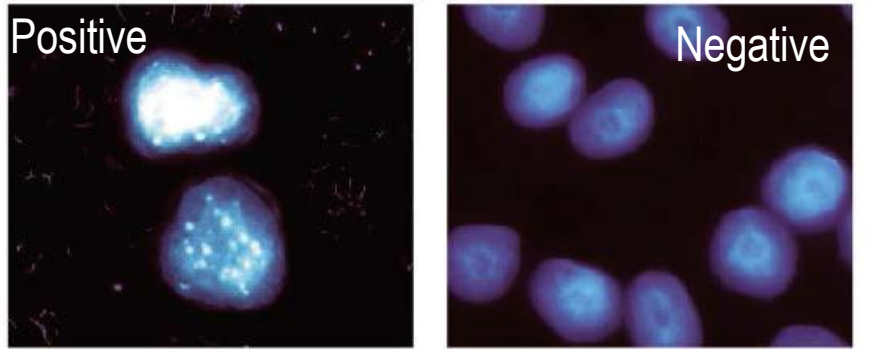
Add 0.5 mL of test sample (culture supernatant) and incubate for 3 to 6 days

Fix the cells, stain them with benzimidazole fluorescent stain, and observe using microscope (x 400-600)

The result is positive when 5 or more cells out of 1000 cells are observed to have small extra-nuclear fluorescence spots.

Preparation of indicator cells is necessary

Test takes 4 to 7 days



Source: Jikken Recipe by Sigma-aldrich

Experience and skills are necessary to judge positive or negative.

Judgement of the result is difficult as this is not a method specifically for mycoplasma.

1-7. Method C: Nucleic Acid Amplification Technique (NAT methods)

<Test method>
Extract DNAs from the sample and amplify them using mycoplasma-specific primers to detect mycoplasma.

Under Japanese Pharmacopoeia 17th Edition…

- Validation methods for NAT technique are presented
  ⇒As far as validation is performed, various methods can be used

- Can be used as an alternative to Method A and Method B
  ⇒If sufficient sensitivity is confirmed through proper validation, the method can be used as an alternative to Method A and Method B

  Alternative to Method A: 10CFU/mL is detectable for all 7 mycoplasma strains
  Alternative to Method B: 100CFU/mL is detectable for all 7 mycoplasma strains.

Nucleic Acid Amplification Technique can be used as a rapid mycoplasma testing method that has superior sensitivity and specificity, as far as proper validation is performed.

2-2. Product outline (Packaging specification)

- Paid samples of Prevrsion (product code: 69201), a mycoplasma gene detection kit, are on sale
- We have agreed on delivery specification of Preversion with the manufacture (NTS) (The manufacturer performs manufacturing, packaging, and labeling of the kit).
- 1 kit contains 48 tests, can be stored in a cool place (2-8℃). Valid for 6 months from the date placed on the market (ultimate goal is 12 months).

<Package presentation>

Mycoplasma gene detection kit Preversion (48 tests)

[Appearance]

(1)

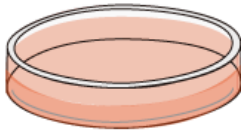
(2)

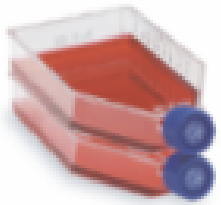
[Constituent reagents]
(1) Test strip A (white) x 6
(2) Test strip B (transparent) x 6
(3) Positive control (2 x 10^3copies/μL) x 1
(4) Negative control (DNase Free Water) x 1
(5) Flat cap x 6

(3) (4) (5)

2-3. Test method outline

Following is a rough outline of mycoplasma testing using this kit





Sample: cell culture

⇒Cell substrate used for the development of medical products

Cell/tissue-based medical products, etc.

Centrifugal concentration

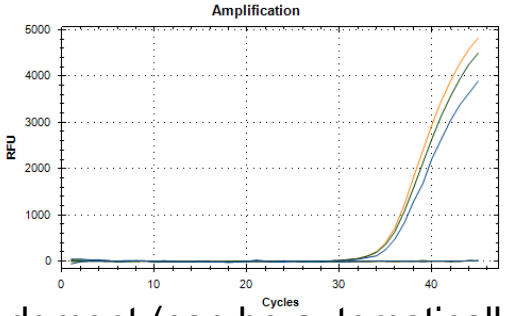
Extraction of nucleic acid

⇒The kit by QIAGEN is recommended

Reagent preparation (detection)

⇒Mycoplasma gene detection kit

Real-time PCR

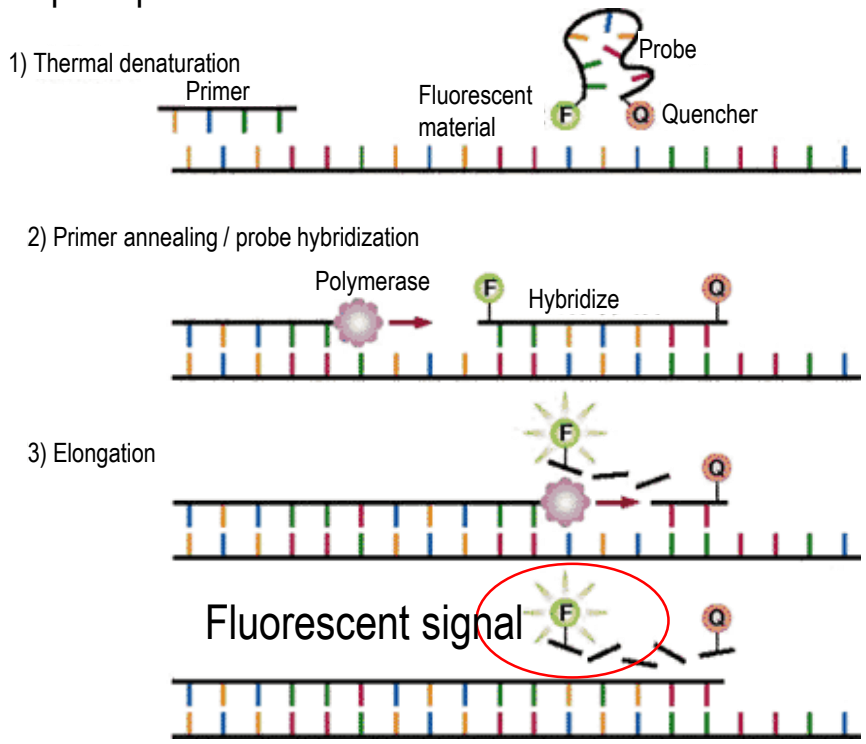


Judgment (can be automatically judged using analysis software)

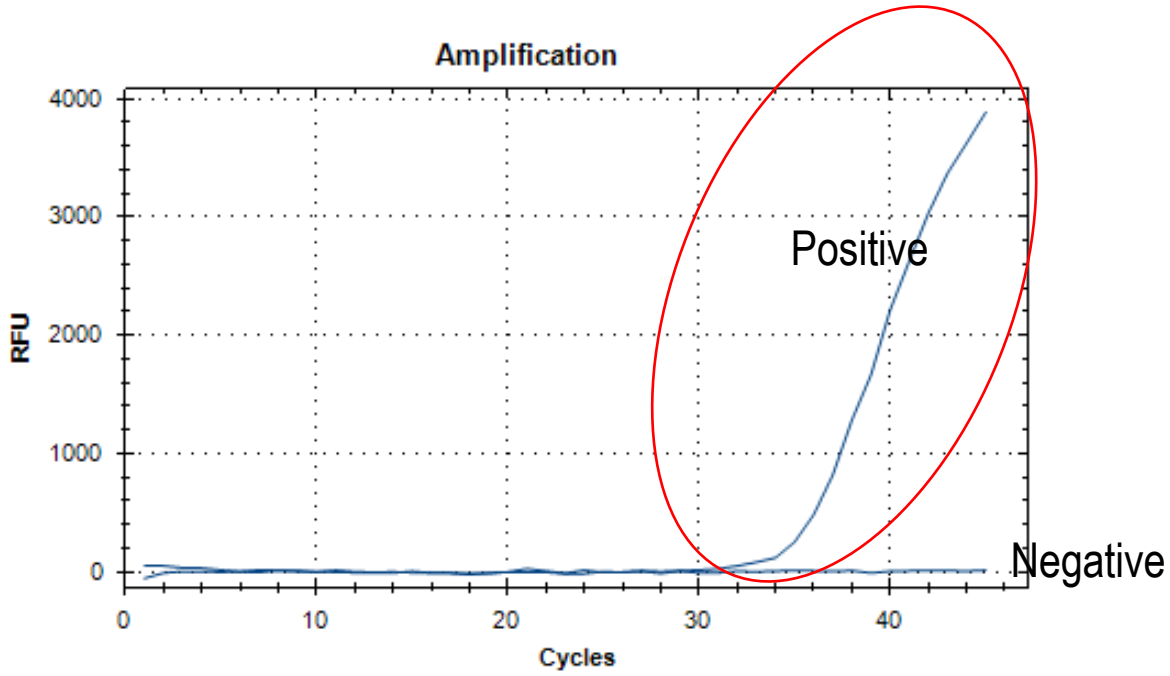
2-4. Real-time PCR

This kit detects mycoplasma nucleic acid by real-time PCR

TaqMan probe method



Source: TAKARA website



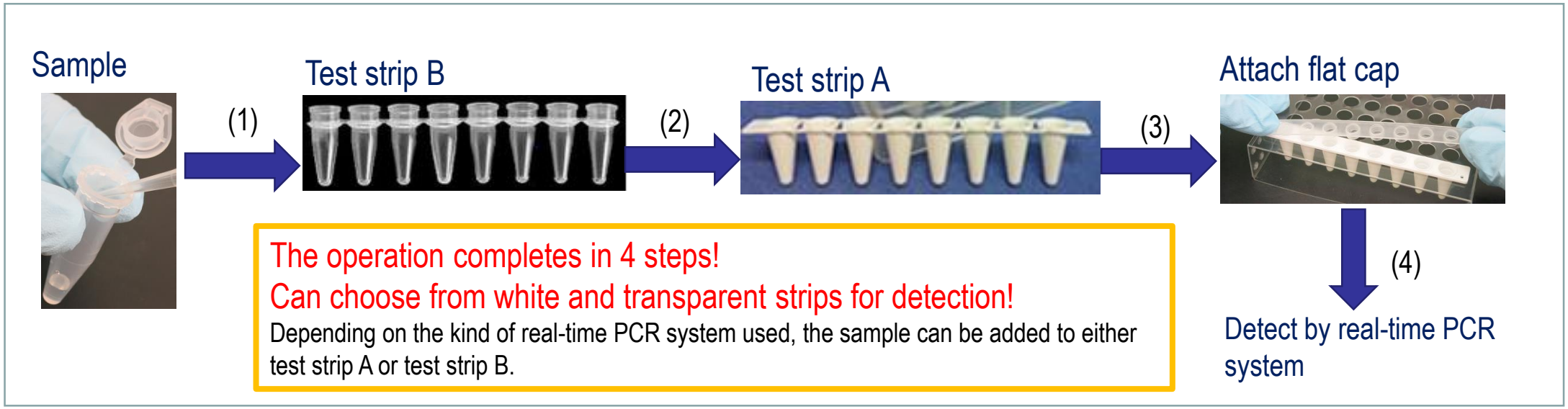
Probes are degraded during PCR and a fluorescent signal is emitted.
By detecting the fluorescent signal, amplification of nucleic acid can be captured.

2-5. How to use the kit (reagent preparation)

- Preparation of reagent is not necessary because dried reaction reagent is set in the reaction tube for each test
- As a potent amplification enzyme is used, detection can complete within one hour from the start of amplification

<Usage method (example)>

(1) Add 25μL of the DNA detection sample at the right concentration to the test strip B
(2) After dissolving the solid reagent in the test strip B by pipetting, move all to the test strip A
(3) After dissolving the solid reagent in the test strip A by pipetting, attach the flat cap
(4) Detect by real-time PCR system

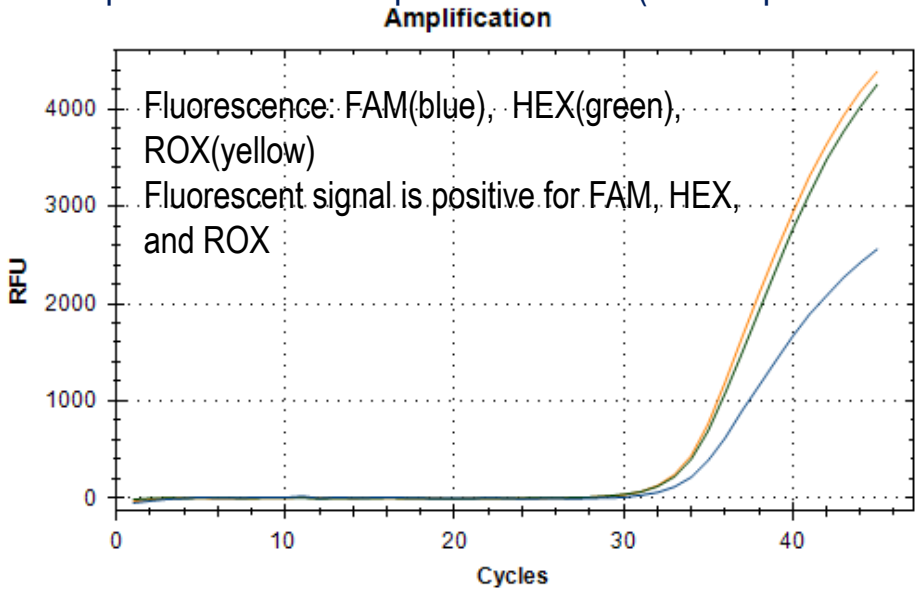


2-6. Product feature ~ Special positive control~

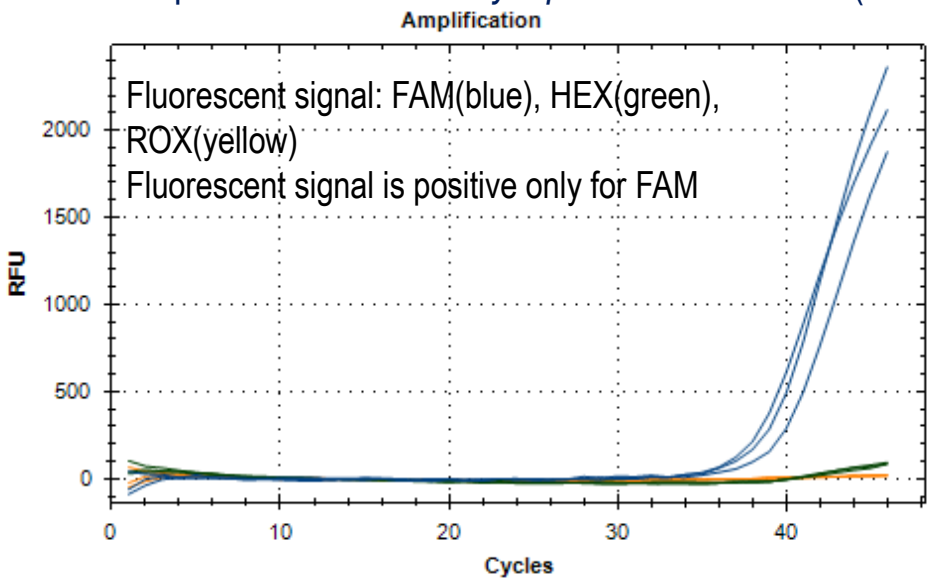
■ False-positive result due to contamination of negative sample by positive control is identifiable

<Example of detection of positive control>

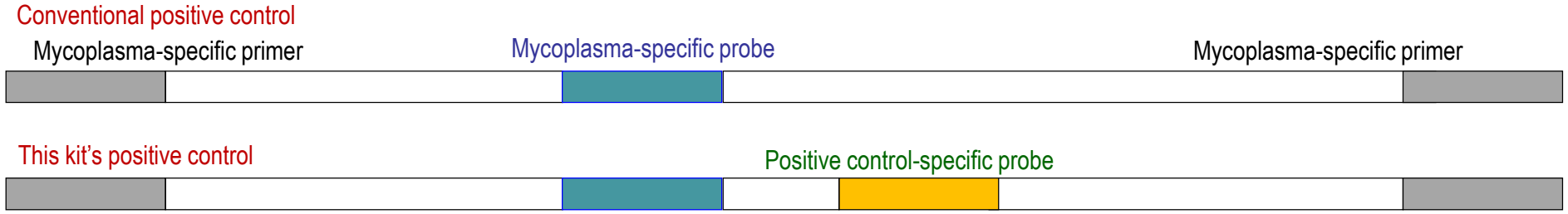
Amplification curve of positive control (1000copies/reaction)



Amplification curve of *mycoplasma fermentans* (10cfu/mL)



<Design of positive control>

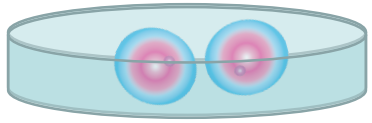


2-7. Comparison between commercially available kits ~basic specification~

Product name	MycoFinder (newly marketed kit)	MycoTOOL Mycoplasma real-time PCR Kit	MycoSEQ Mycoplasma Real-Time PCR
Sold by	Shimadzu Diagnostics Corporation	Roche	ABI
JP compliance	Compliance with JP17 is intended	Compliance with EP is intended	Compliance with JP 17 is intended
Principle	Real-time PCR method	Real-time PCR method	Real-time PCR (SYBR) method
No. of tests / kit	48	About 160	100
Reagent composition	-Strip A: 6 strips -Strip B: 6 strips ・Positive Control: 8vial ・Water: 8vial *PCR reaction reagent is solidified in the strip	・Recovery Control: 1vial(200μL) ・PCR Master: 5vial ・UNG: 1vial (180μL) ・PCR Enhancer: 1vial (180μL) ・Detection Mix(Probe): 1vial (220μL) ・Detection Mix Recovery Control(Probe):1vial (140μL) ・Positive Control: 1vial (800μL) ・Water: 2vial (1mL)	・Primer Mix: 1tube(325μL) ・Negative Control: 1tube(1000μL) ・SYBR Master mix: 2tube(1000μL) ・DNA Control: 1tube(400μL) *Water is not included
Sensitivity	10cfu/ml	10cfu/ml	1-10copies/tube
Specificity	Theoretically 142 species	140 species	90 species
Contamination measure	Identification of positive control against test sample is possible	UNG system	None
Internal control	None	GAPDH	None
Storage temperature	**Cool place (2-8℃)**	-20℃	-15～-25
Time	**2 hours (in case of a potent PCR)**	5 hours	Unknown
Measurement device	Short type only (White and transparent are available)	Universal	Universal

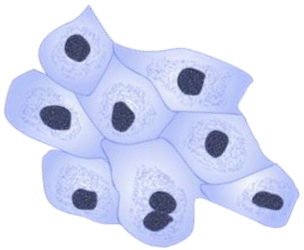
2-8. Use example of the kit

This kit can detect mycoplasma in cell culture ⇒ It can be used not only for the testing of final products, but also for the *voluntary inspection during culture*.



Cell culture starts

Culture



Products such as cells

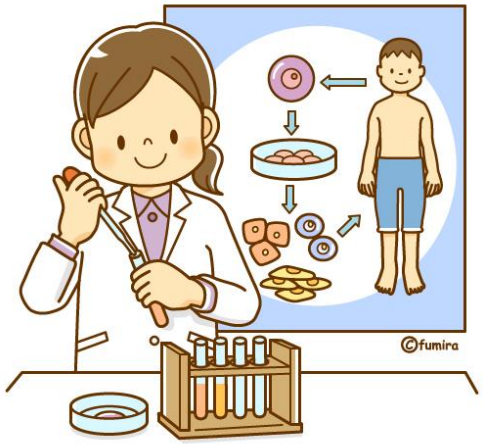
Take a sample from culture solution

Voluntary inspection

⇒Monitor the amount of mycoplasma in culture solution

Mycoplasma testing (Compliant with JP17)

*To implement PCR, pre-validation is necessary.



3. Performance evaluation of the kit and validation to comply with JP

3-1. Why validation is necessary?

Do users have to perform full-validation at their own facilities?

⇒The revision proposal states, “when a commercially available kit is used for NAT testing, the full validation data from the manufacturer can be used as an alternative to the validation data by the user.”

If the maker’s validation data is available, the user does not have to perform full-validation

But!

The differences in cell types of the sample, extraction method, detection device, etc. may affect the sensitivity and reproducibility. Therefore a performance validation need to be performed at the user facility.

Users need to confirm that the same validation result as the one presented by the maker can be obtained at their own facilities.

3-2. Method C (NAT method) validation outline

■The JP17 requires a proper validation be performed.

<Outline of validation method>

(1) Validation of NAT method

✓ Specificity: Detection of 7 species

✓ Detection sensitivity

Mycoplasma reference strains for which characteristic analysis has been performed and concentration (CFU or number of copies, etc.) has been clarified or strains authorized by the international standards have to be used.

Sensitivity for detecting mycoplasma contained in cell suspension has to be evaluated.

The test has to detect mycoplasma with a probability of more than 95%.

✓ Robustness of the analysis method

The method has to be proved reliable under normal use.

(2) When NAT method is used as an alternative to Method A or Method B

✓ Equivalence test

Alternative to Method A: 10CFU/mL is detectable for all 7 strains of mycoplasma

Alternative to Method B: 100CFU/mL is detectable for all 7 strains of mycoplasma

3-3. Validation implementation plan

Details of kit validation is as follows.

<Details of validation>

	Item	Result	Detail
Revised JP	Specificity	Pass	It has to be confirmed that nucleic acids derived from the 7 reference strains for which sequence analysis has been performed are detectable.
	Detection sensitivity	Pass	1, 10, and 100CFU/mL have to be tested to confirm that 10CFU/mL is detectable with a probability of 95% or more.
	Robustness	Under evaluation	Tolerance range has to be evaluated by increasing/decreasing the sample amount from the defined 25uL.
	Equivalence (1)	Pass	It has to be confirmed that the seven strains are detectable at 10CFU/mL .
	Equivalence (2) (culture method)	Pass	It has to be confirmed that the samples used for sensitivity test (10CFU/mL) are also detectable by culture method.
Additional data	Device evaluation	Under evaluation	Shimadzu Corporation device is evaluated.
	Safety test	Under evaluation	Safety test for 6 month is to be performed in March (Up to 4 month data has been obtained.)

3-4. Validation conditions

Conditions of the kit validation are as follows.

<Materials and devices used>

DNA extraction kit: DNeasy Blood & Tissue Kit(QIAGEN)
Real-time PCR system: CFX96(Bio-Rad)
Cell: CHO-DG44 cells (1 x 10^6 cells/mL)
Mycoplasma reference strains: see below

<Reference strain information>

GC values and CFU values of the reference strains prepared in our facility used for evaluation are shown below. GC values and CFU values were measured in accordance with the National Institute of Health Sciences method*.

Strain name	Origin	No. of genomic copies (copies/mL)	CFU (CFU/mL)	GC/CFU
M. pneumonieae	NBRC14401	6.0E+08	2.9E+07	20.7
M. hyorhinis	NBRC14858	1.4E+09	4.2E+08	3.4
A. laidlawii	NBRC14400	3.9E+09	3.5E+08	11.2
M. fermentans	NBRC14854	1.3E+10	3.3E+09	3.9
M. arginini	ATCC23838	4.1E+09	2.2E+09	1.8
M. orale	NBRC14477	1.3E+10	8.3E+08	16.3
M. salivarium	NBRC14478	4.2E+09	2.5E+09	1.7

*Health Labour Sciences Research Grant 2014 Research Report: Evaluation of the safety of innovative drugs against viruses and infectious agents

3-5. Specificity test

■To confirm that 7 types of test strains of mycoplasma are specifically detected, following two experiments were conducted.

Performed PCR using the genomic DNAs from the 7 reference strains as templates

Performed sequence analysis of the PCR product

↓

The DNA sequence of the PCR product has more than 99% equivalence with mycoplasma gene it drives from.

→ It was confirmed that the kit's primer can amplify mycoplasma gene.

Performed sensitivity test using the DNA from the sample (cell suspension + mycoplasma reference strains)

↓

10CFU/mL was detected for the 7 strains.

→ Mycoplasma was specifically detected even when cell-derived DNAs exist.

It was confirmed that this kit *can specifically detect the 7 mycoplasma species*.

3-6. Sensitivity test (Equivalence test)

- Here is the result of the sensitivity test using the serial dilution.

  Sensitivity is to be calculated by statistical analysis.

Detection limit for *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

Detection limit for *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

Detection limit for *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

Detection limit for *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

Detection limit for *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

Detection limit for *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

Detection limit for *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

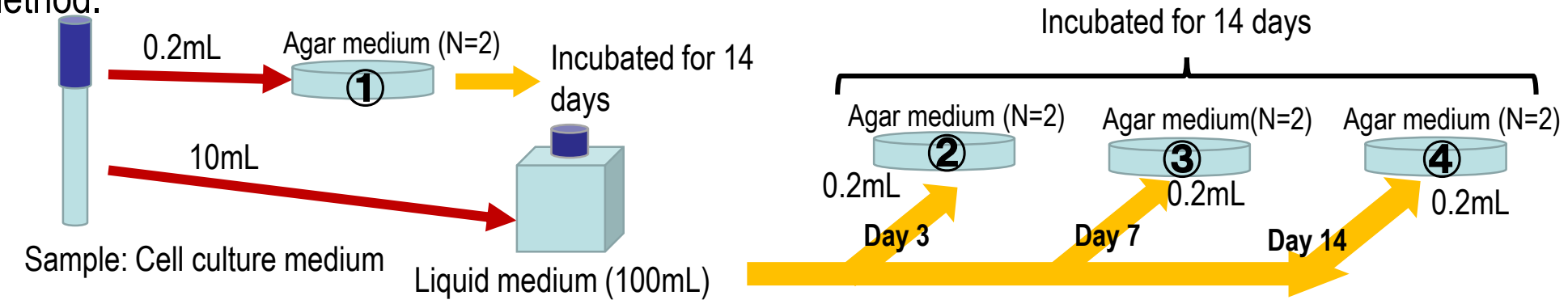
3-7. Equivalence test (NAT technique)

The sensitivity test result showed that the 7 test strains specified by JP17 are detectable at 10-CFU/mL with a probability of 95% or more.⇒The method meets conditions required of an alternative to Method A (culture method)

Sample		1st test	2nd test	3rd test	Total	Detection rate
M. arginini	Reference strains (10 CFU) + cell suspension	8/8	8/8	7/8	23/24	95.8%
M. fermentans		8/8	8/8	7/8	23/24	95.8%
M. hyorhinis		8/8	8/8	8/8	24/24	100.0%
M. pneumoniae		8/8	8/8	8/8	24/24	100.0%
M. orale		8/8	7/8	8/8	23/24	95.8%
M. salivarium		7/8	8/8	8/8	23/24	95.8%
A. laidlawii		8/8	7/8	8/8	23/24	95.8%
Negative sample	Cell suspension			0/24		

3-8. Equivalence test (culture method)

Using the same sample as the sensitivity test (cell suspension + reference strains), detection test was performed by culture method.



Strain name	Inoculated amount	Liquid medium	Plate medium (sampling from liquid medium) Day 1	Day 3 (2)	Day 7 (3)	Day 14 (4)
M.pneumoniae	10CFU/mL (100CFU/bottle)	ww＋	−	−	＋	＋
M.hyorhinis		＋	＋	＋	＋	＋
A.laidlawii		＋	＋	＋	＋	＋
M.fermentans		＋	＋	＋	＋	＋
M.arginini		＋	＋	＋	−	−
M.orale		＋	＋	＋	−	−
M.salivarium		＋	−	＋	＋	＋

It was confirmed that all 7 sample strains yield positive result at 10 CFU/mL.

⇒This shows that *the sensitivity test and the culture method have the same sensitivity*.

3-9. Additional data ~Cross reactivity test~

■No cross-reactivity was observed for 25 types of bacteria, 8 types of funguses, and 3 types of mammalian cells.

<Cross-reactivity>

25 types of bacteria

Bacteroides vulgatus
Bacillus subtilis
Clostridium acetobutylicum
Clostridium kluyveri
Clostridium sporogenes
Escherichia coli
Enterococcus faecalis
Gluconacetobacter xylinus
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

9 types of funguses

Cryptococcus neoformans
Candida albicans
Mucor circinelloides
Cunninghamella echinulata
Rhizomucor pusillus
Absidia corynbifera
Scedosporium prolificans
Pneumocystis carinii

3 types of mammalian cells

Raji Cell (human)
Mouse T lymphocyte (mouse)
CHO cell (hamster)

The genome used for the test is 1ng/reaction*

*Equivalent to about 2 x 10^5copies assuming the average genome size of the bacteria is 5 x 10^6bp