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Belief & Relief, LiliF Diagnostics MDx Kit
Instruction For Use
English (영문, 英語)

e-Myco™ VALiD Mycoplasma PCR Detection Kit

RUO Research Use only
REF 25239 Σ 48 -20℃

Test for the detection of Mycoplasmas by PCR analysis

This kit is covered by patents owned by Abbott Molecular Inc.
(US Pat. No. 5,851,767 and its foreign counterparts)

BACKGROUND INFORMATION

The maintenance of contamination-free cell lines is essential to cell-based research such as biopharmaceutical production, cell therapy and tissue engineering. Mycoplasma is often not visible and does not respond to antibiotics, and therefore it is a major issue that requires monitoring and early detection. Up to 30~85% of cell cultures may be contaminated with mycoplasmas, the main contaminants being the species *M. orale, A. laidlawii, M. arginini* and *M. hyorhinis.* Although these mycoplasmas do not usually kill contaminated cells, they are difficult to detect and can cause a variety of effects on cultured cells, including changes in metabolism growth, viability and morphology, thereby altering the phenotypic properties of the host cells. Traditional detection methods use direct culture method to detect contaminating organisms. But the culture-based methods are time-consuming, requiring as much as 28 days, very laborious and difficult to interpret. Thus recently, it is a trend that PCR-based detection method may be adopted to standard protocol replacing direct culture method including E.P. 2.6.7 directive and drug regulating agencies worldwide.

The e-Myco™ VALiD Mycoplasma PCR Detection Kit is composed a set of primers those are specific for the highly conserved mycoplasma 16S-rRNA coding region including *M. pneumoniae, M. agninini, M. hyorhinis, M. fermentans, M. orale* and *A. laidlawii.* The kit is design to detect the presence of mycoplasma that might contaminate biological materials such as cultured cells. Also, the kit can be performed in 3 hours, can detect sensitively until 10 CFU/ml and includes internal control for verifying a PCR run as well as positive control DNA.

Each tube of the e-Myco™ VALiD Mycoplasma PCR Detection Kit provides all-in-one system(FastMix technology), which means all components for PCR is already pre-aliquoted in each PCR tube. All you need to do is just to add template and distilled water for PCR.

CHARACTERISTICS

- **Ready to use** : This e-Myco™ VALiD Mycoplasma PCR Detection Kit contains all the components for the PCR reaction. You just add template DNA or samples.
- **Time Save** : Replace traditional 28 days culture testing with the kit less than 33 hours
- **Smart (internal control and 8-MOP)** : Internal control system embedded in the product prevents misjudgment that possibly arises from an erroneous PCR test. And the kit can eliminate carry-over contamination with 8-MOP activation.
- **Steady (Broad Species Detection)** : You can detect common cell culture-infecting species of mycoplasma and also other various species of mycoplasma (See Technical Guide).
- **Stage-up (Sensitive and reliable)** : The performance of Mycoplasma Detection meets European Pharmacopoeia Guidance and Regulation of drug regulating agencies. This test is suitable for release testing and in-process control. It can replace culture and indicator cell tests.

INTENDED USE

- For Research Use Only, Not for use in diagnostic procedures.
- in-process monitoring for the presence of Mycoplasma

e-Myco™ VALiD Mycoplasma PCR Detection Kit is developed, designed, and sold for research purpose only. It is not intended to be used for human or animal diagnosis of diseases. Do not use internally or externally in humans or animals. Prior to using it for other purposes, the user must validate the system in compliance with the applicable law, directives, and regulations.

REQUIREMENTS INSTRUMENT

- Pipettes and pipette tips (aerosol barrier)
- Thermal cycler
- Disposable gloves
- G-spin™ Total DNA Extraction Mini Kit
- Vortex mixer
- Heat block

DESCRIPTION

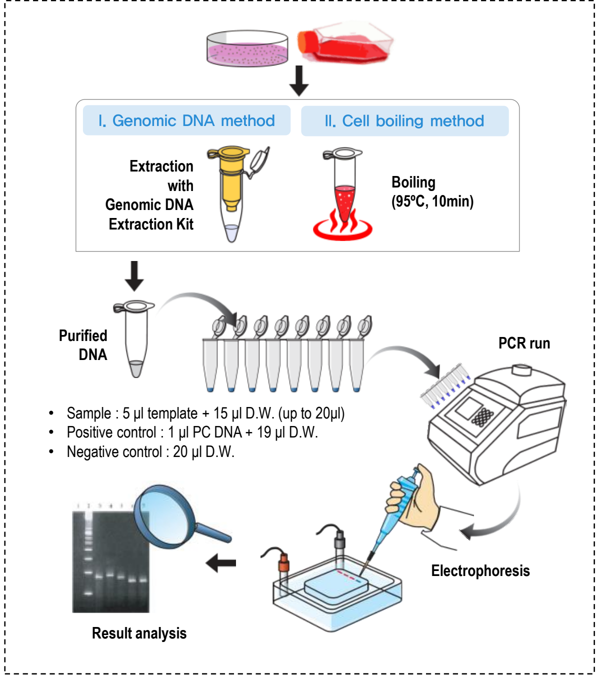
- e-Myco™ VALiD Mycoplasma PCR Premix : Blue colored pellet in PCR Strip
- Control DNA : Colorless and transparent liquid
- DNase/RNase Free Water : Colorless and transparent liquid

KIT CONTENTS, PACKAGING / STORAGE INFORMATIONS

No	Contents	Composition	25239
1	e-Myco™ VALiD Mycoplasma PCR Premix	< 0.01% Hot start Taq DNA Polymerase < 0.01% dATP, dTTP, dGTP, dCTP < 0.005% Mycoplasma Primers, Internal Control < 0.001% 8-MOP (dissolved in DMSO)	48T
2	Control DNA	< 0.01% recombinant DNA included partial 16S sequence of *M. hyorhinis*	25 µl x 3Tubes
3	DNase/RNase Free Water	No template control < DNase/RNase Free Water	1 ml x 1Tube

- Storage condition : Store the product at below -20℃ after receiving.
- Expiration : e-Myco™ VALiD Mycoplasma PCR Detection Kit can be stored for up to 18 months without showing any reduction in performance and quality under appropriate storage condition. The expiration date is labeled on the product box.

OVERVIEW OF MYCOPLASMA DETECTION



SAMPLE PREPARATION

※ Protocol Ⅰ : Genomic DNA Extraction Method

- PCR inhibiting substances may accumulate over time in cell culture medium.
- Medium with more than 10~12 % serum has inhibitory effects on downstream application such as PCR. Moreover, phenol red, a routine material in cell culture medium, is likely to cross-react and thus interfering the signals in PCR.
- These negative effects can be overcame by using the G-spin™ Total DNA Extraction Mini Kit for Sample preparation.
- For this reason, it is recommended to isolate genomic DNA from samples purely to ensure accuracy and repeatability of analysis.

1. **Prepare Gram negative bacteria sample.**
2. **Transfer 1 ~ 2 ml cultured bacteria cell into 2 ml tube.**
3. **Pellet bacteria by centrifugation for 1 min at 13,000 rpm, and discard supernatant. Resuspend completely the cell pellet with remnant supernatant by tapping or vigorously vortexing.**
4. **Add 200 µl Buffer CL, 20 µl Proteinase K and 5 µl RNase A Solution into sample tube and mix by vortexing vigorously.**
5. **incubate lysate at 56℃ using preheated heat block or water bath for 10 ~ 30 min.**
6. **When lysis is completed, add 200 µl of Buffer BL into upper sample tube and mix thoroughly. Then incubate the mixture at 70℃ for 5min.**
7. **Centrifuge the sample tube at 13,000 rpm for 5 min to remove un-lysed tissue particles. Then carefully transfer 350 ~ 400 µl of the supernatant into a new 1.5 ml tube (not provided).**
8. **Add 200 µl of absolute ethanol into the lysate, and mix well by pulse vortex. After mixing, briefly centrifuge the 1.5 ml tube to remove drops from inside of the lid.**
9. **Carefully apply the mixture from step 7 to the Spin Column (in a 2 ml Collection Tube) without wetting the rim, close the cap, and centrifuge at 13,000 rpm for 1 min. Discard the filtrate and place the Spin Column in a new 2 ml Collection Tube (additionally supplied).**
10. **Add 700 µl of Buffer WA to the Spin Column without wetting the rim, and centrifuge for 1 min at 13,000 rpm.**
11. **Add 700 µl of Buffer WB to the Spin Column without wetting the rim, and centrifuge for 1 min at 13,000 rpm. Discard the flow-through and place the Column into a new 2.0 ml Collection Tube (additionally supplied), Then again centrifuge for additional 1 min to dry the membrane. Discard the flow-through and Collection Tube altogether.**
12. **Place the Spin Column into a new 1.5 ml tube (not supplied), and add 30-100 µl of Buffer CE directly onto the membrane. Incubate for 1 min at room temperature and then centrifuge for 1 min at 13,000 rpm to elute.**

※ Protocol Ⅱ : Boiling Method

1. **Prepare cell suspensions from the test cell culture in a 1.5 ml tube. Then count cell numbers by general counting methods. You need at least $5x10^4$ cells per test.**
   Note : Strong mycoplasma infections are detected in as little as 10~100 cells, while weak infections require cells over 5,000~50,000 cells. You can dilute the template according to the infection rates you suspect. We recommend that you perform the PCR reaction after preparing serial dilutions of the straight supernatant to obtain optimal results.
2. **Transfer the counted cells (over $5x10^4$ cells) to a 1.5 ml tube. Spin the tube in a microcentrifuge for 10~15 seconds. Carefully decant the supernatant.**
3. **Resuspend the cells in 1 ml of sterile PBS or DPBS solution for washing.**
4. **Spin the tube in a microcentrifuge for 10~15 seconds. Carefully decant the supernatant.** [Option] Repeat this wash step once more.
5. **Resuspend the cell pellets in 100 µl of sterile PBS or DPBS solution.**
   Note : If you want the best result, use of PBS solution is better than Tris (10 mM, pH 8.5), TE (10 mM Tris, 0.1 mM EDTA), or autoclaved DW.
6. **Heat the samples at 95 ℃ for 10 min, and vortex for 5-10 sec. Then, centrifuge for 2 min at 13,000 rpm with a tabletop centrifuge (at RT).**
7. **Transfer an aliquot of the heated supernatant to a fresh tube. This supernatant will be used as the template in the PCR.**

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PCR TEST PROTOCOLS

※ Precautions before Testing

- Leave it at 4°C or room temperature for thawing. Do not leave it at room temperature more than 1 hour.
- Use clean, disposable gloves when performing the assay and make sure that the work area is clean prior to starting the assay setup.
- All procedures must be done on a clean bench that should be cleaned with 70% alcohol or 10% household bleach (Na-hypochlorite) after use. The samples used should be kept separate. If discarded, it is considered to be a biological hazardous substance after high-pressure sterilization and discarded.

※ Test Procedure

1. **Prepare appropriate number of e-Myco™ VALiD Mycoplasma PCR Premix tubes.**
   ⚠ An appropriate number of tubes means the combination of two tubes in the number of samples, which includes a positive control and a negative control.
2. **Add 15 µl of DNase/RNase-free water into the RT-PCR Pre-mixture tube.**
3. **Add 5µl of DNA sample to each of strip tubes.**
4. **For positive and negative confirmation, use 1 µl of positive control or DNase/RNase Free water as a test sample. Then, adjust the reaction volume to 20 µl.**
5. **Dissolve the blue pellet by pipetting or vortexing.**
   ⚠ The pellet is easily dissolved, by letting the mixture stand at R.T. for 1-2 minutes after adding water.
6. **Perform PCR reaction of samples as the below process using thermal cycler.**

PCR Condition		Temp	Time
Initial denaturation		94 ℃	1 min
X 40 Cycle	Denaturation	94 ℃	30 sec
	Annealing	60 ℃	20 sec
	Extension	72 ℃	1 min
Final extension		72 ℃	5 min

7. **For analysis by electrophoresis, use 5 µl of each tube.**
8. **PCR products should be discarded after UV irradiation (10 min) to prevent carry-over contamination.**
   ⚠ Contamination of DNA is a serious problem of PCR. Please discard PCR products after UV irradiation (365 nm) to prevent carry-over contamination.

TECHNICAL INFORMATION

※ Interpretation

e-Myco™ VALiD Mycoplasma PCR Detection Kit provides the detection of various Mycoplasma species with high sensitivity and specificity. For the validation of each PCR reaction, it includes internal control in each PCR Premix tube. The interpretation of experimental results is as follows.

Band location : Mycoplasma – 260 ~ 280 bp, Internal control – 170 bp

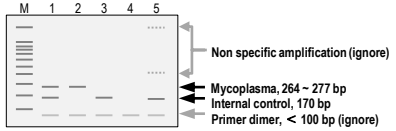

Fig. 1. (ex) Results Interpretation of e-Myco™ VALiD Mycoplasma PCR Detection Kit

Lane	1	2	3	4	5
Mycoplasma	Positive	Positive	Negative	Unknown	Negative
Results	Valid	Valid (a lot of target DNA amount)	Valid (Negative, below 10 cfu/ml)	Invalid, Retest (Poor reaction condition or low quality of template DNA)	Valid (low quality of template DNA or template degradation, ignore the non specific bands or primer dimer band)

※ Specificity test

e-Myco™ VALiD Mycoplasma PCR Detection Kit is design to specifically detect only Mycoplasma spp. and provides high specificity resulted from no cross reactivity with other similar microorganisms. Fig.2 shows the evaluation data of e-Myco™ VALiD Mycoplasma PCR Detection Kit, suggesting that both internal control (app. 180 bp) and target bands (app. 270 bp) were detected. However, in cases of negative control(20 ng of non-mycoplasma bacterial genomic DNA, lanes 2~7) and no template control (lane N), only negative control band was detected

Lane	Name	ATCC No.
1	*Mycoplasma hyorhinis*	17981D-5
2	*Clostridium perfringens*	13124D-5
3	*Streptococcus mutans*	700610D-5
4	*Lactobacillus plantarum*	BAA-793D-5
5	*Mobiluncus mulieris*	35240D-5
6	*Gardnerella vaginalis*	49145D-5
7	*Haemophilus ducreyi*	700724D-5
8	*DNase/RNase Free Water*	

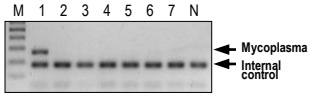

Fig. 2. Specificity of e-Myco™ VALiD Mycoplasma PCR Detection Kit
Lane M, SiZer™-100 DNA Marker; **Lane 1,** *Mycoplasma hyorhinis*; **Lane 2,** *Clostridium perfringens*; **Lane 3,** *Streptococcus mutans*; **Lane 4,** *Lactobacillus plantarum*; **Lane 5,** *Mobiluncus mulieris*; **Lane 6,** *Gardnerella vaginalis* **Lane 7**,*Haemophilus ducreyi*; **Lane N,** No template control.

※ Analytical Sensitivity

e-Myco™ VALiD Mycoplasma PCR Detection Kit is an eligible kit for the efficient detection of *Mycoplasma spp*. contamination with high sensitivity in the culture.
To identify the analytical sensitivity, the genomic DNA from cell cultured 6 Mycoplasma spp. were purified. The sensitivity according to the DNA copy number was investigated after purifying gDNA from each cultured Mycoplasma spp.

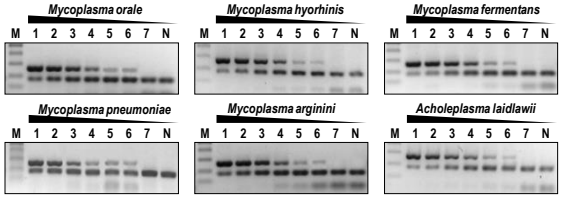

Fig. 3. Analytical Sensitivity of e-Myco™ VALiD Mycoplasma PCR Detection Kit
LaneM, SiZer™-100 DNA Marker; **Lane 1,** 1×10^6 cfu/ml of gDNA; **Lane 2,** 1×10^5 cfu/ml of gDNA; **Lane 3,** 1×10^4 cfu/ml of gDNA; **Lane 4,** 1×10^3 cfu/ml of gDNA; **Lane 5,** 1×10^2 cfu/ml of gDNA; **Lane 6,** 10 cfu/ml of gDNA; **Lane 7**, 1 cfu/ml of gDNA; **Lane N,** No template control.

※ Comparison with direct plating method

e-Myco™ Mycoplasma PCR Detection Kit shows much higher sensitivity than conventional culture plate method, based on the direct comparison of PCR result done by this kit with the conventional colony counts, using 10-folds diluted Mycoplasma culture supernatant.

[Direct plating : *A. laidlawii*]

Dilution rate	Colony No.	Cell conc. (cfu/ml)
10^{-7}	92 ± 2	0.9×10^9
10^{-8}	12 ± 3.5	1.2×10^9
10^{-9}	1.3 ± 0.5	1.3×10^9

[PCR Detection : *A. laidlawii*]

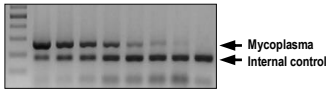

Fig. 4. Comparative test of e-Myco™ VALiD Mycoplasma PCR Detection Kit with direct plating method.
LaneM, SiZer™-100 DNA Marker; **Lane 1,** 10^{-3} diluted *A. laidlawii*; **Lane 2,** 10^{-4} diluted *A. laidlawii*; **Lane 3,** 10^{-5} diluted *A. laidlawii*; **Lane 4,** 10^{-6} diluted *A. laidlawii*; **Lane 5,** 10^{-7} diluted *A. laidlawii*; **Lane 6,** 10^{-8} diluted *of A. laidlawii*; **Lane 7**, 10^{-9} diluted *A. laidlawii*; **Lane N,** No template control.

※ SPECIES DETERMINATION BY SEQUENCING ANALYSIS

- The sequence of amplified PCR products differs slightly from species to species. You can determine approximately the Mycoplasma species by sequencing analysis with the following primers. Please refer to the phylogenetic table on the next page. For more detailed species analysis, you should perform additional PCR reactions with your designed primers.
- We list only the Forward primer sequences. Please synthesize the primer, and then analyze by general sequencing methods.
- Sequencing primer sequences : AGGAT TAG ATA CCC TGG TAG TC-3' (20 mer)
  ⚠ The PCR primers used in this kit differ from the sequencing primers. We do not list the PCR primer sequences contained in this kit.

※ TROUBLESHOOTING GUIDE

Symptoms	Possible Causes	Comments & Suggestions
No Target band in positive reaction	Check internal control band	• If internal control band is seen, PCR has been performed properly; it is not a problem of the product.
	Check the quality or concentration of template	• If the PCR reaction is inhibited by impurities included in DNA preparation, the use of diluted DNA as a template may be helpful. • Whereas the signals of internal control (app. 160 bp length) are shown, if the target band is not shown, it indicates that the sample is not infected by Mycoplasma
	Check a PCR machine	• The problem can be caused by the PCR machine. Please check the temperature and make sure to check that the machine is working properly.
No internal control band	Check template concentration	• Competition can occur by using high concentrated DNA template. Please repeat the PCR with a diluted template. If the concentration of template is above 50 ng, the signal of internal control may be disappeared by competition with the template.
	Check the quality of template (possibility of contamination with PCR inhibitors)	• If the PCR reaction is inhibited by impurities included in DNA preparation, the use of diluted DNA as a template may be helpful. If there is no internal control band, please inquire with our technical support staff.
	Check the storage condition of product.	• Keep appropriate preservation conditions
Presence of amplified product in the negative control	Check contamination of D.W.	• D.W. can be contaminated. Perform PCR again with fresh sterile water
	Check contamination of lab instruments and other environments	• We recommend that you use filter tips to reduce contamination and that you use a pipette after sterilization. All procedures should be done in sterilized conditions.
Poor resolution on agarose gel	Low gel concentration	• We recommend to use a 1.5~2% agarose gel. • Check the resolution comparing with DNA marker
	Short running time	• We recommend that electrophoresis is performed for 40 min at 100 V/14 cm using a 6 cm long 2% agarose gel.

ORDERING INFORMATION

Product Name	Amount	Cat. No.
G-spin™ Total DNA Extraction Mini Kit	50 Col.	17045
SiZer™-100 DNA Marker	0.5 ml	24073
e-Myco™ VALiD² Mycoplasma PCR Kit	48 tubes	IP21432

※ References

1. Santer et al., C1q Deficiency Leads to the Defective Suppression of IFN-α in Response to Nucleoprotein Containing Immune Complexes, J Immunol., 2010, 185(8), 4738–4749.
2. J.-L.Ku et al., Establishment and characterization of 13 human colorectal carcinoma cell lines : mutations of genes and expressions of drug-sensitivity genes and cancer stem cell markers, Carcinogenesis, 2010, 31(6), 1003–1009
3. Pettersson et al., Sequence Analysis of 16S rRNA from Mycoplasmas by Direct Solid-Phase DNA Sequencing, Appl. Environ. Microbiol., 1994, 60 (7), 2456-2461.
4. European Pharmacopoeia. 6.1, section 2.6.7 Mycoplasma; Revised January 2008.
5. Guidance on the Validation of Nucleic Acid Amplification Tests for the Detection of Mycoplasmas, KFDA, Revised April 2012.

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EXPLANATION OF SYMBOLS

Manufactured by | Attention | Consult Instructions For Use
Manufacturing date | Expire date | Keep away from sunlight
Sufficient for tests | Do not reuse | Storage temperature limitation
Batch number | In vitro diagnostic | Product number
LOT | IVD | REF

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