Attachment 3

Prepared in November 2015 (1st edition)

Mycoplasma testing

Mycoplasma Gene Detection Kit Preversion

Instruction

——— Background to Development and Product Features ———

This kit offers a real-time PCR-based test to detect mycoplasma genes extracted from cell cultures. As this kit contains two types of strips in which all the reaction reagents are solidified, reagent preparation is very easy and result can be obtained quickly with easy operation.

——— General Precautions ———

1) Read this instructions carefully before using the kit. Follow the operational procedures specified in this instructions when using the kit. Reliability of the kit cannot be guaranteed when it is used without following the specified operational procedures or for any purposes other than those specified in the instructions.
2) All samples should be treated as infectious, and a user has to wear protections (glasses, gloves, masks, etc.) and operate very carefully when using this kit.
3) This reagent is intended for research use only. Do not use this kit for other purposes.

——— Forms and configurations, etc. <Kit components> ———

Constituent reagents

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

——— Purpose of Use ———

Detection of mycoplasma genes extracted from cell culture.

——— Operational Precautions ———

1) If probes or primers in the reaction reagents are degraded by nuclease, the test cannot yield accurate detection. Nuclease contamination can occur not only from test tools and instruments, but also from sweats and saliva of the user. Please operate carefully.
2) To prevent contamination of the sample, it is recommended that the work space for sample preparation/DNA extraction and the work space for reaction reagent preparation are physically separated. If it is difficult, please perform UV irradiation or clean-up of the work space before moving on to the next operation.
3) After taking test strips out of the aluminum bag, please seal the bag well so that the remaining test strips will be stored safely. Please avoid light as they contain fluorogenic reagent.
4) When using the test strips, be careful not to drop them from a height or subject them to strong physical shock.
5) Mix the positive control well and spin it down before use.
6) Be sure to use new disposable tips to minimize the risk of contamination between samples when dispensing reagents.
7) When handling a real-time PCR system, follow the instructions of the system.
8) As this kit offers a rea-time PCR-based test in which amplification and detection are performed at the same time, there is no need to perform electrophoresis of PCR products. Do not take PCR products out of the test strips as it can cause contamination.

———Directions of Use/Dosage (Operation Method)———

[Necessary tools]

Please make the following tools and equipment ready as necessary.

Mixer, micro pipet, filter tip (sterilized, DNase/RNase Free), centrifugal separator, centrifuge tube, real-time PCR system

[Operation method]

1. DNA extraction method

Following is the extraction method using QIAGEN DNeasy Blood and Tissue Kit.

Other commercially available DNA extraction kits can also be used, but it is recommended that validation of extraction efficiency, etc. be performed in advance.

1) Add 20μL of Proteinase K and 200μL of Buffer AL to a 200μL of cell culture or a 200μL of cell suspension prepared by concentrating cell culture by centrifugation, and mix well by a mixer.
2) Incubate at 56°C for 15 minutes.
3) Add 200μL of 99 % ethanol and mix well by a mixer.
4) Move all into spin column and centrifuge at 6,000 × g (8,000 rpm) for 1 minute.
5) Place the spin column into a new collection tube and add 500μL of Buffer AW1. Centrifuge at 6,000 × g (8,000 rpm) for 1 minute.
6) Place the spin column into a new collection tube and add 500μL of Buffer AW2. Centrifuge at 20,000 × g (14,000 rpm) for 3 minutes.
7) Place the spin column into a new collection tube and centrifuge the empty column at 20,000 × g (14,000 rpm) for 1 minute.
8) Confirm that here is no remaining liquid in the column.
9) Place the spin column into a separately prepared centrifuge tube and add 100μL of Buffer AE. Incubate at room temperature (15 to 25°C) for 5 minutes.
10) Centrifuge at 6,000 × g (8,000 rpm) for 1 minute and prepare a reagent using the obtained sample.

2. How to prepare a reagent

1) Take the necessary quantity of test strip A and test strip B out of the aluminum bag.
2) Add 25μL of the sample prepared by DNA extraction to test strip A, and pipet gently for 20 times to dissolve the solidified reagent. Dilute the sample as necessary before use.
3) Move all the dissolved sample in test strip A into test strip B, and pipet gently for 20 times to dissolve the solidified reagent.
4) Attach flat cap to test strip B, and detect by real-time PCR.

*When detection is performed using a white tube, first add the sample to test strip B, dissolve, and then move it into test strip A.

3. Detection by real-time PCR

Following is the protocol for BioRad CFX96.

1) Set the fluorescence detection wavelength of the real-time PCR system to FAM + ROX (or FAM + HEX). Each fluorescence is used for detection of the following.
   (1) Detection of mycoplasma: FAM
   (2) Detection of positive control: FAM + ROX (or FAM + HEX)

*Before starting real-time PCR, fluorescent filter settings need to be adjusted to detect several fluorescence. When BioRad CFX96 is used, set the Scan Mode to 'All channels' to read data from all the installed filters.

2) Set the program of the real-time PCR in reference to the following standard. Please refer to the instructions of your real-time PCR system for how to change settings.

95℃ 10Sec
98℃ 3Sec
60℃ 1Sec
} 45cycle

——— How to Judge the Measurement Result ———

Judgment is made using the analysis software for the real-time PCR system.

BioRad CFX96 uses regression method and Background subtracted Curve Fit mode for analysis.

——— Precautions Regarding Judgment———

1) When FAM + ROX is set as fluorescence detection wavelength for real-time PCR, fluorescence intensity of FAM will increase for a mycoplasma-positive sample, while fluorescence intensity of FAM and ROX will increase at almost the same cycle quantification value (Cq value) for a positive control. Fluorescence intensity will not increase for a negative control or negative sample.
2) When FAM + HEX is set as fluorescence detection wavelength for real-time PCR, please be aware of any potential leakage of fluorescence from FAM into HEX.

——— Performance ———

1. Sensitivity/accuracy test

When standard plasmids of *M. arginine, M. orale,M. salivarium,M. hyorhinis,M. fermentans, M. pneunoniae,* and *Acholeplasma laidlawii* (10 copies / test) are used as positive control, and negative control (DNase Free Water) is used as negative control for the test using this kit, all the positive controls will yield positive result, and all the negative controls will yield negative result.

———Precautions for Use and Handling ———

1 . Precautions for handling (hazard control)

All samples should be treated as infectious, and a user has to wear protections (glasses, gloves, masks, etc.) and operate very carefully when using this kit to avoid risk of infection.

2. Precautions for use

1) Avoid freezing this product and store under the specified storage condition (2 to 8 ℃).
2) Do not use any expired reagents.
3) Do not mix reagents of different production numbers.
4) Do not use this kit for other purposes than those specified here.

3. Precautions for Disposal

1) To prevent contamination, any test strips used for PCR should be sealed in doubled plastic bags with the flat cap on, and disposed as medical waste following the waste disposal rules. Do not autoclave PCR products as they may scatter.
2) Wen disposing of reagents, equipment, etc. used for the test, process them as medical waste, industrial waste, or infectious waste following the relevant regulations such as Waste Management and Public Cleaning Act and Water Pollution Prevention Act, etc.

———Storage/Expiration Date———

[Storage method]

Store at 2 to 8°C.

[Expiration date]

3 months from the production date

*Expiration date is indicated on the outer package and on the container label.

——— Contact ———

[Contact for logistics inquiries]
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[Contact for scientific inquiries]
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