Cell Culture Mycoplasma Detection by PCR

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[Abstract] DNA was extracted from the supernatant of each sample. After PCR-amplification of mycoplasma DNA, detection was performed by gel electrophresis. The PCR primers were designed to cover the consensus sequences that can detect all types of mycoplasma species.

Materials and Reagents

1. Ampli Taq Gold (Life Technologies, Invitrogen™, catalog number: 4338856)
2. Sodium acetate
3. Ethanol
4. Phenol
5. $MgCl_2$
6. PCR buffer
7. Sodium acetate
8. dNTPs
9. Agarose gel
10. TE-saturated phenol
11. Ethidium bromide
12. DDW
13. Stock solution A (see Recipes)
14. Master mixture A (see Recipes)

Equipment

1. 1.5 ml Eppendorf tube
2. Centrifuges
3. Micropipette

Procedure

A. Preparation of template DNA

1. From cultured supernatant (in the case of cells)
   a. The sample aliquots (600 µl/sample) are obtained from the supernatant of the sample cells in the chamber. We use one 1.5 ml Eppendorf tube per sample.
   b. Add same amount of TE-saturated phenol (600 µl) to each eppendorf tube and mix vigously by vortex for several seconds.
   c. Centrifuge at 15,000 rpm for 5 min at room temperature (RT).
   d. Transfer the 400 µl supernatant to a new eppendorf tube and add 10 µl of 3 M sodium acetate.
   e. Mix well and spin down the aliquots.
   f. Add 2.5 times volume of absolute ethanol (1 ml), mix well, and keep at -80 °C for 15 min.
   g. Centrifuge at 15,000 rpm for 10 min at 4 °C.
   h. Discard the supernatant by micropipette and rinse with 80% ethanol.
   i. Centrifuge at 15,000 rpm for 10 min at 4 °C.
   j. Discard the supernatant by micropipette completely and air-dry.
   k. Dissolve in 40 µl DDW by vigorous vortexing and use this as the template DNA for PCR.
2. From frozen ampule of sample cells
   a. Thaw the frozen ampule at RT.
   b. Open the ampule and take 600 µl of cell suspension to a 1.5 ml Eppendorf tube.
   c. Add same amount of TE-saturated phenol (600 µl) to each eppendorf tube and mix vigorously by vortex for several seconds. From this step, perform all the same procedures described in A. 2-11.

      Note: In this protocol the final 40 µl dissolved solution becomes quite viscous because of containing large amounts of genomic DNA from the sample cells. Therefore, vortex mixing is needed for a longer time.

B. PCR

Reaction mixture per sample (per one tube)

DDW	29.75 µl
Stock solution A	15.0 µl
Template DNA	5.0 µl
Ampli Taq Gold(5 U/µl)	0.25 µl
Total	50.0 µl

1. Beforehand we make "Stock solution A" and "Master mixture A" for 10 tubes as recipes. Distribute 45 µl of "Master mixture A" to each tube.
2. Add 5.0 µl of template DNA, mix well, and spin down.
3. Perform PCR cycling by the thermal cycler machine as following schedule:

   95 °C 9 min

   94 °C 30 sec*

   55 °C 2 min * *30 cycles repeated

   72 °C 2 min *

   72 °C 5 min
4. Store the samples at 4 °C.

C. Agarose gel electrophoresis

1. 10 µl of PCR products are loaded onto 2% agarose gel.
2. Stain the gel with ethidium bromide (0.1 µg/ml) for 10 min and take photograph under UV light.

   Note: If you know that the sample was highly contaminated by mycoplasma, you can use the supernatant from cultured sample cells directly for the PCR reaction mixture. In this case 3-5 µl of the supernatant will be applicable to the reaction mixture of PCR without all the above sample preparation procedures.

Recipes

1. Stock solution A (15 µl for 1 sample)

10x PCR buffer	5 µl
$MgCl_2$ (25 mM)	4 µl
dNTPs (each 2.5 mM)	4 µl
Primer F1 (10 pmol/µl)	1 µl
Primer R1 (10 pmol/µl)	1 µl
Total	15 µl

2. Master mixture A (45 µl per tube, for 10 samples)

DDW	312.4 µl
Stock solution A	157.5 µl
Ampli. Taq Gold	2.6 µl
Total	472.5 µl

References

1. Harasawa, R., Mizusawa, H., Nozawa, K., Nakagawa, T., Asada, K. and Kato, I. (1993). Detection and tentative identification of dominant mycoplasma species in cell cultures by restriction analysis of the 16S-23S rRNA intergenic spacer regions. *Res Microbiol* 144(6): 489-493.

