

## Detection of Mycoplasma Contaminations

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### Summary

Mycoplasma contamination of cell lines is one of the major problems in cell culture technology. The specific, sensitive, and reliable detection of mycoplasma contamination is an important part of mycoplasma control and should be an established method in every cell culture laboratory. New cell lines as well as cell lines in continuous culture must be tested in regular intervals. The polymerase chain reaction (PCR) methodology offers a fast and sensitive technique to monitor all cultures in a laboratory. The technique can also be used to determine the contaminating mycoplasma species.

The described assay can be performed within 3 h, including sample preparation, DNA extraction, performing the PCR reaction, and analysis of the PCR products. Special precautions necessary to avoid false-negative results resulting from inhibitors of the *Taq* polymerase present in the crude samples and the interpretation of the results are also described.

**Key Words:** Bacteria; cell lines; contamination; mycoplasma; PCR.

### 1. Introduction

#### 1.1. *Mycoplasma Contaminations of Cell Lines*

Acute contaminations of cell lines are frequently observed in routine cell culture and can often be attributed to improper handling of the growing culture. These contaminations can usually be detected by the turbidity evolving after a short incubation time or by routine observation of the culture under the inverted microscope. In addition to these obvious contaminations, other hidden infections can occur consisting of mycoplasmas, viruses, or cross-contaminations with other cell lines. Although known for many years and despite the multitude of publications dealing with mycoplasma infections of cell cultures, a high proportion of scientists are not aware of the potential contamination of cell cultures with mycoplasmas. As seen in our cell repository, more than 25%

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of the incoming cell lines are infected with mycoplasmas, and in most cases, the depositor was not aware of this. Whereas in the early years of cell culture, bovine serum was one of the major sources of infections, nowadays mycoplasmas seem to be mainly transferred from one infected culture to another by using laboratory equipment, media, or reagents that came into contact with infected cultures. This culture hopping is concordant with the occurrence of cross-contaminations with a proved incidence of 16% plus an estimated number of unknown cases (**1**). Thus, methods for the detection, elimination (*see* Chapter 3), and prevention of mycoplasma contaminations should belong to the basic panel of cell culture techniques applied.

The term “Mycoplasma” is usually used as a synonym for the class of Mollicutes that represents a large group of highly specialized bacteria and are all characterized by their lack of a rigid cell wall. Mycoplasma is the largest genus within this class. Because of their small size and flexibility, these bacteria are able to pass through conventional microbiological filters. Mycoplasmas can be seen as commensals, because their reduced metabolic abilities cause a relatively long generation time, which is in the range of that of cell lines, and they do usually not overgrow or kill the eukaryotic cells. However, their influence on the biological characteristics of the eukaryotic cells is manifold and almost every experimental or production setting can be influenced. The identification of infecting mycoplasmas shows that only a limited number of about seven *Mycoplasma* and *Acholeplasma* species from human, swine, and bovine hosts occur predominantly in cell cultures, and no species specificity can be observed. Additionally, a couple of mycoplasma species were shown to enter the eukaryotic cells actively and to exist intracytoplasmic (**2**). Hence, sensitive methods need to be established and frequently employed in every cell culture laboratory to detect mycoplasma contaminations.

## **1.2. Mycoplasma Detection**

The biological diversity of mycoplasmas and their close adaptation to cell cultures renders it very difficult to detect all contaminations in one general assay. A large spectrum of approaches have been described to detect mycoplasma in cell cultures. Many of these methods are lengthy, complex, and not applicable in routine cell culture (e.g., electron microscopy, biochemical and radioactive incorporation assays, etc.) or are restricted to specific groups of mycoplasmas. Molecular biological methods were the first to be able to detect all the different mycoplasma types in cell cultures, regardless of their biological properties, with a relatively low effort in terms of time and labor (**3**).

Polymerase chain reaction (PCR) provides a very sensitive and specific option for the direct detection of mycoplasmas in cell cultures. PCR combines many of the features that were covered earlier by different assays: sensitivity,

specificity, low expenditure of labor, time, and costs, simplicity of the assay, objectivity of interpretation, reproducibility, and documentation of the results. On the other hand, a number of indispensable control reactions must be included in the PCR assay to avoid false-negative or false-positive results. A comparison of the PCR method with other well-established assays (DNA/RNA hybridization, microbiological culture) showed that the PCR assay is a very robust, efficient, and reliable method for the detection of mycoplasmas (4).

The choice of the primer sequences is one of the most crucial decisions. Several primer sequences are published for both single and nested PCR (see **Note 1**) and with narrow or broad specificity for mycoplasma or eubacteria species. In most cases, the 16S rDNA sequences are used as target sequences, because this gene contains regions with more and less conserved sequences. This gene also offers the opportunity to perform a PCR with the 16S rDNA or an RT-PCR (reverse transcriptase-PCR) with the cDNA of the 16S rRNA (see **Note 2**) (5). Here, we describe the use of a mixture of oligonucleotides for the specific detection of mycoplasmas. This approach reduces significantly the generation of false-positive results resulting from possible contamination of the solutions used for sample preparation and the PCR run and from other materials with airborne bacteria. Nevertheless, major emphasis should be placed on the preparation of the template DNA, the amplification of positive and negative control reactions, and the observance of general rules for the preparation of PCR reactions. One of the main problems concerning PCR reactions with samples from cell cultures is the inhibition of the *Taq* polymerase by unspecified substances. To eliminate those inhibitors, we strictly recommend that the sample DNA be extracted and purified by conventional phenol-chloroform extraction or by the more convenient column or matrix-binding extraction methods. To confirm the error-free preparation of the sample and PCR run, appropriate control reactions have to be included in the PCR. These comprise internal control DNA for every sample reaction and, in parallel, positive and negative as well as water control reactions. The internal control consists of a DNA fragment with the same primer sequences for amplification, but it is of a different size than the amplicon of mycoplasma-contaminated samples. This control DNA is added to the PCR mixture in a previously determined limiting dilution to demonstrate the sensitivity of the PCR reaction. In this chapter, detailed protocols are provided to establish the PCR method for the monitoring of mycoplasma contaminations in any laboratory.

## 2. Materials

1. PBS (phosphate-buffered saline): 140 mM NaCl, 27 mM KCl, 7.2 mM Na<sub>2</sub>HPO<sub>4</sub>, 14.7 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.2. Autoclave 20 min at 121°C to sterilize the solution.

2. 50X TAE (Tris–acetic acid–EDTA): 2 M Tris base, 5.71% glacial acetic acid (v/v), 100 mM EDTA. Adjust to pH of approx 8.5.
3. DNA extraction and purification system (e.g., phenol–chloroform extraction and ethanol precipitation, or DNA extraction kits applying DNA binding matrices).
4. GeneAmp 9600 thermal cycler (Applied Biosystems, Weiterstadt, Germany).
5. *Taq* DNA polymerase (Qiagen, Hilden, Germany).
6. 6X Loading buffer: 0.09% (w/v) bromophenol blue, 0.09% (w/v) xylene cyanol FF, 60% glycerol (v/v), 60 mM EDTA.
7. Primers (any supplier) (*see Note 3*):

5' primers (Myco-5'):

cgc ctg agt agt acg **twc** gc

tgc ctg **rgt** agt aca ttc gc

cgc ctg agt agt atg ctc gc

cgc ctg ggt agt aca ttc gc

3' primers (Myco-3'):

gcg gtg tgt aca **ara** ccc ga

gcg gtg tgt aca **aac** ccc ga

(**r** = mixture of g and a; **w** = mixture of t and a)

Primer stock solutions: 100  $\mu$ M in dH<sub>2</sub>O, stored frozen at –20°C. Working solutions: mix of forward primers at 5  $\mu$ M each (Myco-5') and mix of reverse primers at 5  $\mu$ M each (Myco-3') in distilled water (dH<sub>2</sub>O), aliquoted in small amounts (i.e., 25 to 50- $\mu$ L aliquots), and stored frozen at –20°C.

8. Internal control DNA: can be obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) (4). A limiting dilution should be determined experimentally by performing a PCR with a dilution series of the internal control DNA.
9. Positive control DNA: a 10-fold dilution of any mycoplasma-positive sample prepared as described in **Subheading 3.1.** or obtained from the DSMZ.
10. Deoxy-nucleotide triphosphate mixture (dNTP mix): mixture contains 5 mM each of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and deoxythymidine triphosphate (dTTP) (Pqlab, Erlangen, Germany) in H<sub>2</sub>O and stored as 50- $\mu$ L aliquots at –20°C.
11. 1.3% Agarose–TAE gel (6).

### 3. Methods

The following subsections describe the sample collection, extraction of the DNA, setting up and performing the PCR reaction, the interpretation of the results, and, in addition, the identification of the mycoplasma species. These techniques can also be used to detect mycoplasma contamination in culture media or other supplements (*see Note 4*).

Every incoming cell culture should be kept in quarantine until mycoplasma detection assays are completed and the infection status is clearly determined.

Positive cultures should either be discarded and replaced by clean cultures or cured with specific antibiotics (*see* Chapter 3). Only definitely clean cultures should be used for research experiments and for the production of biologically active pharmaceuticals. Additionally, stringent rules for the prevention of further mycoplasma contamination of cell cultures should be strictly followed (**1**).

### 3.1. Sample Collection and Preparation of DNA

1. Prior to collecting the samples, the cell line to be tested for mycoplasma contamination should be in continuous culture for several days and without any antibiotics (even penicillin and streptomycin) or after thawing for at least 2 wk. This should assure that the titer of the mycoplasmas in the supernatant is within the detection limits of the PCR assay.
2. One milliliter of the supernatant of adherently growing cells or of cultures with settled suspension cells are taken for the analysis. Collecting the samples in this way, some viable or dead eukaryotic cells are included in the test. This is of advantage, as some mycoplasma strains predominantly adhere to the eukaryotic cells or even invade them. Thus, it is also not necessary to centrifuge the sample to eliminate the eukaryotic cells. The crude cell culture supernatants can be stored at 4°C for a few days or frozen at -20°C for several weeks. After thawing, the samples should be further processed immediately.
3. The cell culture suspension is centrifuged at 13,000g for 5 min. The pellet is resuspended in 1 mL PBS by vortexing.
4. The suspension is centrifuged again and washed one more time with PBS as described in **step 3**.
5. After centrifugation, the pellet is resuspended in 100 µL PBS by vortexing and then heated to 95°C for 15 min.
6. Immediately after lysing the cells, the DNA is extracted and purified by standard phenol–chloroform extraction and ethanol precipitation (**6**) or other DNA isolation methods (*see* **Note 5**).

### 3.2. PCR Reaction

The amplification procedure and the parameters described here are optimized for the use in thin-walled 0.2-mL reaction tubes in an Applied Biosystems GeneAmp 9600 thermal cycler. An adjustment to any other equipment might be necessary (*see* **Note 6**). Amplified positive samples contain high amounts of target DNA. Thus, established rules to avoid DNA carryover should be strictly followed: (1) The places where the DNA is extracted, the PCR reaction is set up, and the gel is run after the PCR should be separated from each other; (2) all reagents should be stored in small aliquots to provide a constant source of uncontaminated reagents; (3) avoid reamplifications; (4) reserve pipets, tips, and tubes for their use in the PCR only and irradiate the pipets frequently by ultraviolet (UV) light; (5) the succession of the PCR setup described below should be followed strictly; (6) wear gloves during the whole

sample preparation and PCR setup; (7) include the appropriate control reactions, such as internal, positive, negative, and the water control reaction.

1. Per sample to be tested, two reactions are set up with the following solutions. Sample only: 1  $\mu\text{L}$  dNTPs, 1  $\mu\text{L}$  Myco-5', 1  $\mu\text{L}$  Myco-3', 1.5  $\mu\text{L}$  of 10X PCR buffer, 9.5  $\mu\text{L}$   $\text{dH}_2\text{O}$ ; sample and DNA internal standard: 1  $\mu\text{L}$  dNTPs, 1  $\mu\text{L}$  Myco-5', 1  $\mu\text{L}$  Myco-3', 1.5  $\mu\text{L}$  of 10X PCR buffer, 8.5  $\mu\text{L}$   $\text{dH}_2\text{O}$ , 1  $\mu\text{L}$  internal control DNA.

For several samples, premaster mixtures can be performed. For the reaction without internal control DNA, three reactions have to be added (for the positive, negative, and the water control reactions), and for the reactions with the internal control DNA, two reactions have to be added for the positive and the negative control reaction (*see* **Notes 7** and **8**). For both premaster mixtures, add also the amounts for an additional reaction to have a surplus for pipetting variations.

2. Transfer 14  $\mu\text{L}$  of each of the pre-master mixtures to 0.2 mL PCR reaction tubes and add 1  $\mu\text{L}$   $\text{dH}_2\text{O}$  to the water control reaction.
3. Prepare the *Taq* DNA polymerase mix (10  $\mu\text{L}$  per reaction, plus one additional reaction for pipetting variations) containing 1X PCR buffer and 1 U *Taq* polymerase per reaction.
4. Set aside all reagents used for the preparation of the master mix. Take out the samples of DNA to be tested and the positive control DNA. Do not handle the reagents and samples simultaneously. Add 1  $\mu\text{L}$  per DNA preparation to one reaction tube that contains no internal control DNA and to one tube containing the internal control DNA.
5. To perform a hot-start PCR, transfer the reaction mixtures (without *Taq* polymerase) to the thermal cyclor and start one thermo cycle with the following parameters: step 1, 7 min at 95°C; step 2, 3 min at 72°C; step 3, 2 min at 65°C; step 4, 5 min at 72°C.

During step 2, open the thermal lid and add 10  $\mu\text{L}$  of the *Taq* polymerase mix to each tube. For many samples, the duration of this step can be prolonged. Open and close each reaction tube separately to prevent evaporation of the samples. Allow at least 30 s after adding the *Taq* polymerase to the last tube and closing the lid of the thermal cyclor for equilibration of the temperature within the tubes and removal of condensate from the lid before continuing to the next cycle step.

6. After this initial cycle, perform 32 thermal cycles with the following parameters: step 1, 4 s at 95°C; step 2, 8 s at 65°C; step 3, 16 s at 72°C plus 1 s of extension time during each cycle.
7. The reaction is finished by a final amplification step at 72°C for 10 min and the samples are then cooled down to room temperature.
8. Prepare a 1.3% agarose–TAE gel containing 0.3  $\mu\text{g}$  of ethidium bromide per milliliter (**6**). Submerge the gel in 1X TAE and add 12  $\mu\text{L}$  of the amplification product (10  $\mu\text{L}$  reaction mixtures plus 2  $\mu\text{L}$  of 6X loading buffer) to each well and run the gel at 10 V/cm.
9. Visualize the specific products on a suitable UV light screen and document the results.

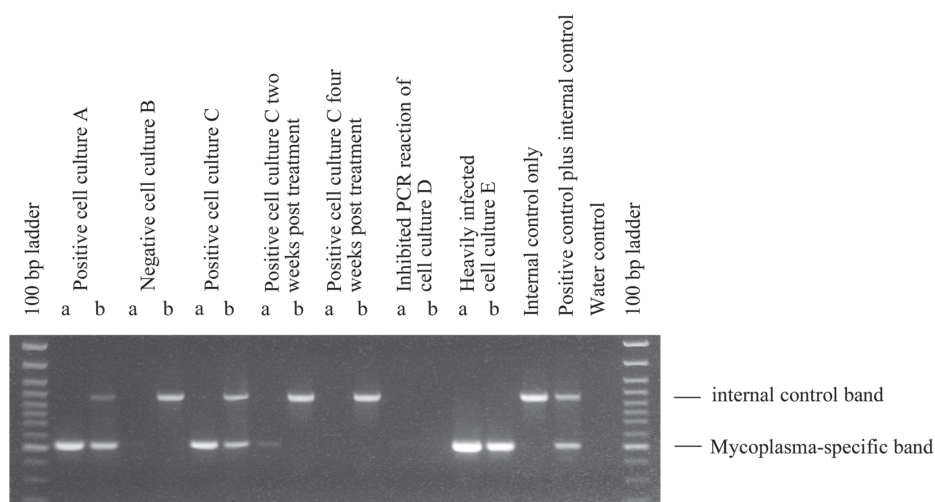


Fig. 1. The PCR analysis of mycoplasma status in cell lines. Shown is an ethidium bromide-stained gel containing the reaction products following PCR amplification with the primer mix listed in the Materials section. Products of about 510 bp were obtained; the differences in length reflect the sequence variation between different mycoplasma species. Shown are various examples of mycoplasma-negative and mycoplasma-positive cell lines. Two paired PCR reactions were performed: one PCR reaction contained an aliquot of the sample only (a) and the second reaction contained the sample under study plus the control DNA as internal standard (b). Cell cultures A, C, and E are mycoplasma positive; cell culture B is mycoplasma negative. The analysis of cell culture D is not evaluable because the internal control was not amplified and no other mycoplasma-specific band appeared in the gel. In this case, the analysis needs to be repeated. Cell line C 2 wk after antibiotic treatment shows a weak but distinctive band in the reaction without internal control. This band results from residual DNA in the medium, because after a further 2 wk of culture, no contamination was detected.

### 3.3. Interpretation of Results

**Figure 1** shows a representative ethidium bromide-stained gel with some samples that produce the following results:

- Ideally, all samples containing the internal control DNA show a band at 986 bp. This band might be more or less bright, but the band has to be visible if no other bands are amplified (*see Note 9*). Otherwise, the reaction might have been contaminated with *Taq* polymerase inhibitors from the sample preparation. In this case, it is usually sufficient to repeat the PCR run with the same DNA solution as previously. It is not necessary to collect a new sample from the cell culture. Even if the second run also shows no band for sample and the internal control, the whole procedure should be repeated.



- Mycoplasma-positive samples show a band at 502–520 bp, depending on the mycoplasma species. In the case of *Acholeplasma laidlawii* contamination and applying the DSMZ internal control DNA, a third band might be visible between the internal control band and the mycoplasma-specific band. This is formed by cross-hybridization of the complementary sequences of the single-stranded long internal control DNA and the shorter single-stranded mycoplasma DNA form.
- Contaminations of reagents with mycoplasma-specific DNA or PCR product are revealed by a band in the water control and/or in the negative control sample.
- Weak mycoplasma-specific bands can occur after treatment of infected cell cultures with antimycoplasma reagents for the elimination of mycoplasma or when other antibiotics such as penicillin–streptomycin are applied routinely. In these cases, the positive reaction might either be the result of residual DNA in the culture medium derived from dead mycoplasma cells or from viable mycoplasma cells present at a very low titer. Therefore, special caution should be taken when cell cultures are tested that were treated with antibiotics. Prior to PCR testing, cell cultures should be cultured for at least 2–3 wk without antibiotics or retested at frequent intervals to demonstrate either a decrease or increase of mycoplasma infection.

### 3.4. Identification of Mycoplasma Species

Although the method described is sufficient to detect mycoplasma contaminations, it might be of advantage to know the infecting mycoplasma species (e.g., in efforts to determine the source of a contamination). This PCR method allows the identification of the mycoplasma species most commonly infecting cell cultures by modified restriction fragment length polymorphism analysis. In case of a contamination detected by PCR, the PCR reaction is repeated in a 50- $\mu$ L volume without the internal control DNA to amplify only the mycoplasma-specific PCR fragment. Per reaction, 8  $\mu$ L of the amplified DNA is directly taken from the PCR reaction and is digested in parallel reactions with the restriction endonucleases *AspI*, *HaeIII*, *HpaII*, and *XbaI* by the addition of 1  $\mu$ L of the appropriate 10X restriction enzyme buffer and 1  $\mu$ L of the restriction enzyme. The mycoplasma species can be determined directly by the restriction pattern (see **Fig. 2**). This analysis allows only the determination of those mycoplasma species that most often (>98%) occur in cell cultures and is not suitable for the global identification of all types of mycoplasma species. Cell culture infections are commonly restricted to about a half dozen mycoplasma species listed in **Fig. 2**.

## 4. Notes

1. Originally, the described method was also designed as nested PCR (7). Here, the second round of PCR was omitted, because in standard applications, no significant differences in the results were observed between one round of PCR only and



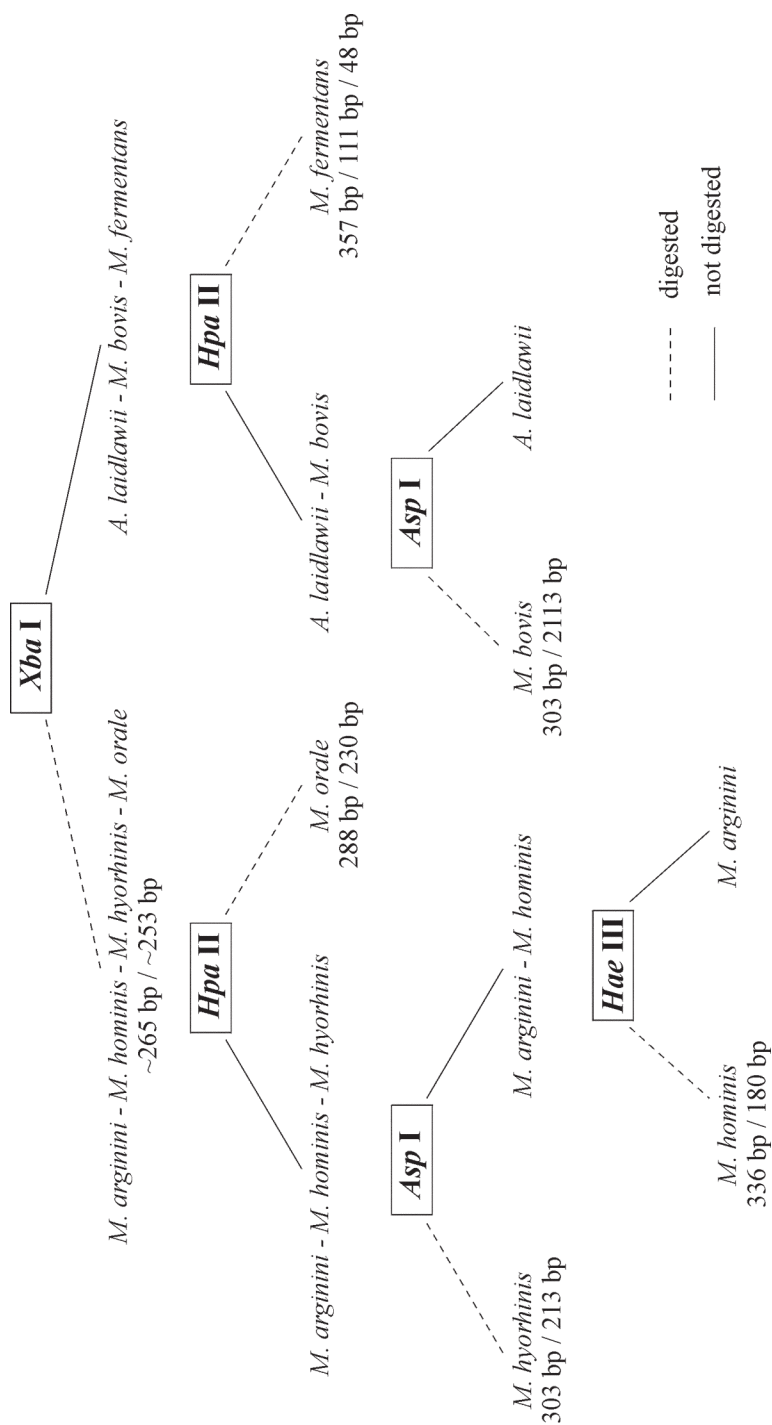


Fig. 2. Flowchart for the identification of the mycoplasma species. Digesting aliquots of the amplified PCR product with the indicated restriction enzymes will result in undigested (solid lines) or digested (dashed lines) fragments of the sizes mentioned below the species names.

nested PCR. Mycoplasma-positive cell cultures were detected as positive in the first round of PCR and negative samples were consistently negative employing nested PCR. Furthermore, applying a nested PCR increases the risk of transmission of first-round PCR products to the reagents used in the second amplification and potentially to those shared with the first round.

2. In this protocol, genomic DNA is used for the PCR reaction. As the primers hybridize to the 16S rRNA, an RT-PCR can also be performed after extracting RNA and preparation of cDNA. RT-PCR might increase the sensitivity of the assay, because the number of rRNA molecules per organism is much higher than the coding gene. Nevertheless, we find that the sensitivity of the described method is high enough for routine applications, and the excess of labor, time, and costs required for RT-PCR protocols is not warranted.
3. The primers can be designed using the degenerated code to incorporate two different nucleotides to form a mixture of two primers. When the forward or reverse primers are mixed and aliquoted for use in the PCR reaction, it must be taken into account that the molarities of the oligonucleotides with mixed bases are reduced by 50%. The primer solutions should be aliquoted into small portions (i.e., 25- $\mu$ L aliquots) and stored frozen at  $-20^{\circ}\text{C}$  to avoid multiple freeze-thawing cycles and to minimize contamination risks.
4. To use this PCR method for the testing of cell culture media or supplements (e.g., fetal bovine serum [FBS]), the sample sizes can be increased and centrifugation performed in an ultracentrifuge.
5. We do not recommend using the crude lysate of the sample for the PCR reaction as described in some publications, because it often contains inhibitors of the *Taq* polymerase and could lead to false-negative results. For convenience and speed of the assay, we apply commercially available DNA extraction/purification kits based on binding of the DNA to matrices and subsequent elution of the DNA. We tested normal phenol-chloroform extraction and subsequent ethanol precipitation, the High Pure PCR Template Preparation Kit from Roche (Mannheim, Germany), the Invisorb Spin DNA MicroKit III from Invitex (Berlin, Germany), and the Wizard DNA Clean-Up System from Promega (Mannheim, Germany). Following the recommendations of the manufacturers, the amplification of the mycoplasma sequences were all similar when the same amounts were used for the elution or resuspension. For screening many samples, the Wizard system works very well with the vacuum manifold.
6. The use of thermal cyclers other than the GeneAmp 9600 might require some modifications in the amplification parameters (e.g., duration of the cycling steps, which are short in comparison to other applications). Also, magnesium, primer, or dNTP concentrations might need to be altered. The same is true if another *Taq* polymerase is used, either polymerases from different suppliers or different kinds of *Taq* polymerase; for example, we found that the parameters described were not transferable to HotStarTaq with a prolonged denaturation step (Qiagen).
7. The limiting dilution of the internal control DNA can be used maximally for 2 or 3 mo when stored at  $4^{\circ}\text{C}$ . After this time, the amplification of the internal control

DNA might fail even when no inhibitors are present in the reaction, because the DNA concentration might be reduced because of degradation or attachment to the plastic tube.

8. Applying the internal control DNA, the described PCR method is competitive only for the group of mycoplasma species that carries primer sequences identical to the one from which the internal control DNA was prepared. The other primer sequences are not used up in the PCR reaction because of mismatches. Usually, one reaction per sample is sufficient to detect mycoplasma in long-term infected cell cultures. However, to avoid the possibility of performing a competitive reaction and of decreasing the sensitivity of the PCR reaction (e.g., after antimycoplasma treatment or for the testing of cell culture reagents), two separate reactions are performed: (1) without internal control DNA to make all reagents available for the amplification of the specific product and (2) including the internal control DNA to demonstrate the integrity of the PCR reaction (see **Fig. 1**).
9. Heavily infected cell cultures might show the mycoplasma specific band, whereas the internal control is not visible. In this case, the mycoplasma target DNA suppresses the internal control, which is present in the reaction mixture at much lower concentrations. The reaction is classified mycoplasma positive (see **Fig. 1**).

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