

Mycoplasma Detection by the Mycoplasma PCR ELISA

MANFRED WIRTH, MARTINA GRASHOFF, LORIN SCHUMACHER, AND HANSJÖRG HAUSER

GBF-Gesellschaft für Biotechnologische Forschung,
Mascheroder Weg 1, 38124 Braunschweig, Germany

Introduction

The most frequent contaminants of animal cell cultures are mycoplasmas. According to recent screenings, about 35% of all cell cultures are contaminated with mycoplasmas (1). Due to their size and lack of a bacterial cell wall, mycoplasmas are able to pass through 0.2 μm filters commonly used for sterile filtration of media and media components. Mycoplasmas cannot be detected by the light microscope. Unlike ordinary bacterial contamination, mycoplasmal infection does not cause turbidity in the cell culture medium, even if growth densities of 10^8 particles/ml are reached. Another prominent feature of mycoplasmas is their resistance to various antibiotics used for the prevention of bacterial contamination in cell culture.

An array of physiological and biochemical parameters are affected by the presence of mycoplasmas in cell culture. Mycoplasmas are known to induce chromosome instability in mammalian cells and to inhibit cell growth. Furthermore, mycoplasmas interfere with several biochemical assays, such as reverse transcriptase assays, as well as proliferation assays based on thymidine incorporation. Keeping these facts in mind, a sensitive, reliable, and regular mycoplasma diagnosis is advisable for laboratories handling animal cell cultures.

Various methods are available for the detection of the five mycoplasma species most commonly found in mammalian cell culture (*M. fermentans*, *M. hyorhinis*, *M. orale*, *M. arginini*, *A. laidlawii*; see references 2,3). These methods are based on microbiological culture, DNA staining with fluorescent dyes, immunobinding, enzymatic or serological tests, ELISA, and/or PCR. The detection methods differ with respect to the ease of detection, speed, sensitivity, and specificity.

We have tested a Boehringer Mannheim nonradioactive PCR ELISA method for the detection of mycoplasmas typical for mammalian cell culture. The method is derived from a "nested PCR" detection method that has been previously established (4). The Boehringer Mannheim Mycoplasma PCR ELISA circumvents the efforts commonly required to prevent carry-over contaminations caused by the second PCR step; the PCR ELISA permits a single-step PCR in which a digoxigenin label is introduced, followed by digoxigenin detection.

Materials and Methods

Buffers and reagents of the Boehringer Mannheim Mycoplasma PCR ELISA were used as supplied with the kit.

Sample 1 preparation

Mycoplasmas were grown, and DNA was isolated essentially as described in reference 4.

Sample 2 preparation

Supernatants from animal cells were centrifuged for 10 minutes at $13,000 \times g$ and 4°C . Supernatants were removed carefully, and 10 μl sterile water and 10 μl of lysis reagent were added to the pellet, which was invisible in some cases. After a 1 h incubation at 37°C , 30 μl of neutralization reagent was added.

Amplification

In a PCR tube, 10 µl of the sample was mixed with 15 µl of sterile water and 25 µl of the ready-to-use PCR master mix. After a preincubation for 5 minutes at 95°C, DNA was amplified in Thermal Cyclers 480 and 9600 (Perkin Elmer) for 40 PCR cycles (strand separation, 30 sec at 94°C; annealing, 30 sec at 62°C; elongation, 1 min at 72°C) followed by a final elongation for 10 minutes at 72°C.

Detection

Ten microliters of the sample was incubated with 40 µl of denaturation reagent for 10 min at room temperature. Hybridization reagent (450 µl), prepared as described by Boehringer Mannheim, was added, and 200 µl samples were transferred to streptavidin-coated microtiter plates, followed by a 3 h incubation at 37°C with constant agitation. After three washes with 250 µl of washing solution per well, 200 µl of peroxidase-conjugated anti-digoxigenin was added to each well and agitated at room temperature for 30 min. Following five washes with 250 µl washing buffer per well, 100 µl of substrate was incubated with each sample well for 5–20 min. Thereafter, 100 µl of stop reagent was added to each well, and the absorbance was subsequently measured at 450 nm in a microtiter plate (ELISA) reader.

Precautions to avoid contamination

To prevent contamination, the recommendations of Kwok *et al.* (5) were followed. The risks of false positives were minimized by separation of pre- and post-PCR steps. Specific pipettes were used for pre- and post-PCR reactions. Several negative controls were processed.

Results and Discussion

The assay procedure is divided into the following steps (Figure 1):

1. For sample preparation, the mycoplasmas are enriched by centrifugation of the cell-free supernatants and then lysed by alkali treatment, and the lysate is neutralized.
2. A single round of PCR is performed, using a set of mycoplasma group-specific primers that bind to conserved regions of the mycoplasma 16S rDNA. PCR is carried out in the presence of digoxigenin-11-dUTP, leading to the

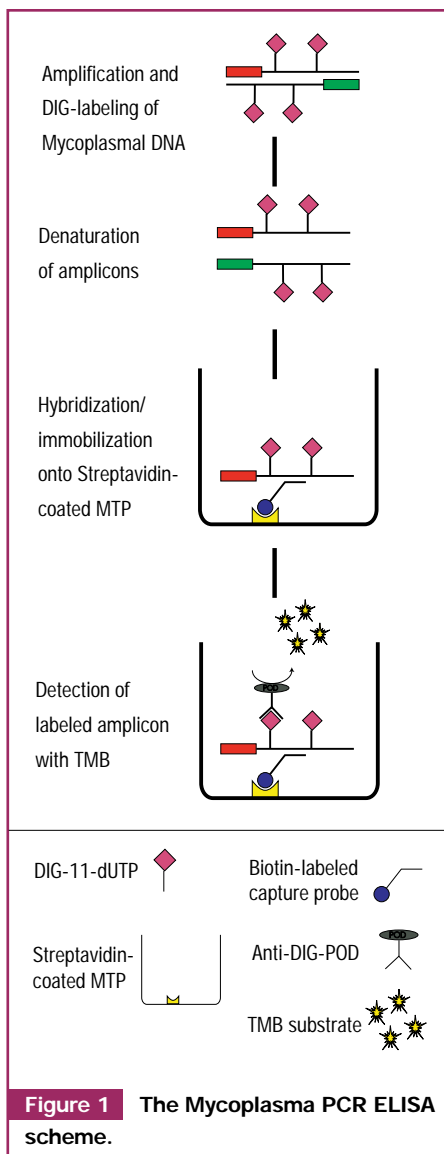


Figure 1 The Mycoplasma PCR ELISA scheme.

incorporation of this label into the amplicon.

3. Mycoplasma-specific DNA is detected after denaturation of the PCR amplicon and subsequent hybridization with a specific, biotinylated capture probe. The probe is complementary to a region within the primer binding sites of the amplified product. The hybrid is immobilized on streptavidin-coated microtiter plates and detected in a well-established immunoreaction using anti-digoxigenin antibodies.

Specificity of the PCR ELISA procedure

The specificity of the assay was determined by using proteinase K/SDS-purified DNAs from 20 chromosomal DNAs of different organisms. Fifteen DNAs were of

mycoplasma origin, including the five most common contaminants of animal cell culture. Five chromosomal DNAs were derived from species that are closely related (*Lactobacillus casei*, *Clostridium perfringens*), more distantly related (*E. coli*), or not related (*Saccharomyces cerevisiae*, mouse *Ltk*⁻ cells) to mycoplasmas.

The Mycoplasma PCR ELISA detected all *Mycoplasma* and *Acholeplasma* species (Table 1). Four out of five negative control DNAs were negative in this assay. A weak positive signal was obtained from *Clostridium perfringens* DNA. However raising the temperature for the annealing step in the “nested PCR” type progenitor of this PCR ELISA assay has been shown to eliminate such cross reaction (4). Based on data derived from the nested PCR detection of mycoplasmas, we anticipate that at least ten more *Mollicutes* species, including *M. pirum*, should be detectable.

Species	Detectable
<i>M. orale</i>	+
<i>M. arginini</i>	+
<i>M. fermentans</i>	+
<i>A. laidlawii</i>	+
<i>M. hyorhins</i>	+
<i>M. salivarium</i>	+
<i>M. gallisepticum</i>	+
<i>M. hominis</i>	+
<i>M. bovis</i>	+
<i>M. californicum</i>	+
<i>M. bovoculi</i>	+
<i>M. PG50 bovine group</i>	+
<i>U. urealyticum</i>	+
<i>M. bovigenitalium</i>	+
<i>M. hyopneumoniae</i>	+
<i>Saccharomyces cerevisiae</i>	-
<i>E. coli</i>	-
<i>Mouse Ltk⁻ cells</i>	-
<i>Lactobacillus casei</i>	-
<i>Clostridium perfringens</i>	(+)

Table 1. Specificity of the PCR ELISA. Average A₄₅₀ values lie between 1 and 2 when mycoplasma DNA is used. Samples are regarded as mycoplasma contaminated if the difference in absorbance between the sample and the negative control is greater than 0.2 A₄₅₀ units.

Sensitivity of the PCR ELISA

Two types of sensitivity assays were performed. First, defined amounts of purified DNA from five mycoplasmal DNAs were used, four of them representing predominant contaminants of cell culture (Table 2). The sensitivity varies, ranging from 1 pg (*M. arginini*, *M. hominis*) to <1 fg (*M. fermentans*, *A. laidlawii*). Since 1 fg approximates 1–3 copies of 16S rDNA, as little as 1–3 mycoplasma particles are detectable in the case of *M. fermentans* or *A. laidlawii*. Provided that suspected animal cells are propagated in the absence of antibiotics, mycoplasma densities can easily reach 10⁸ organisms/ml. Therefore, even a detection limit of 1000 particles/ml is sufficient for routine diagnosis of mycoplasma contamination of cell cultures.

Species	Sensitivity (DNA)
<i>M. fermentans</i>	<1 fg
<i>A. laidlawii</i>	<1 fg
<i>M. orale</i>	10 fg
<i>M. arginini</i>	1 pg
<i>M. hominis</i>	1 pg

Table 2. Sensitivity of the Mycoplasma PCR ELISA using purified mycoplasmal DNA of different species.

Supernatants from naturally infected cell cultures were taken for a second array of sensitivity experiments. In all cases, mycoplasmas were detected – even in dilutions of 10⁵ (Table 3).

Cell type	Contamination with	Detectable by PCR ELISA after dilution of	Microbiological cultivation ^a
SW480	<i>M. arginini</i>	>10 ⁵	+
LA1	<i>A. laidlawii</i>	>10 ⁵	+
Ψ2	<i>M. hyorhinis</i>	>10 ⁵	– ^b

Table 3. Comparison of the Mycoplasma PCR ELISA and the microbiological culture method.

^a Generally, after a dilution of 10³, the detection limit is achieved.
^b In addition, using the DAPI assay, the mycoplasma contamination was not detected.

For further evaluation, the Mycoplasma PCR ELISA was compared to the microbiological cultivation method, which has served for a long time as the “gold standard” for mycoplasma detection methods. In two cases, the PCR ELISA data were confirmed by cultivation. Generally, due to the sensitivity limit, dilutions >10³ of cell culture supernatants were not recommendable for the cultivation method. In one case, microbiological cultivation failed to detect *M. hyorhinis*, most probably since this species is not easily cultivatable.

Taken together, the specificity and sensitivity of the Mycoplasma PCR ELISA are superior to that of known non-PCR mycoplasma assays. The advent of PCR technology combined with nonradioactive hybridization/detection methods facilitates the detection of mycoplasmas. The method combines the features necessary for an elegant detection method. Sample preparation is easy because extraction procedures are avoided. The set up of the PCR reaction is simple, employing a ready-to-use mix. Increased sensitivity and specificity are achieved by a capture hybridization followed by a nonradioactive detection cascade. Also, the appearance of false positives as a result of carry-over is reduced by omitting a second PCR step and gel analysis. However, despite the omission of a second PCR step, precautions should be followed to prevent PCR contaminations (5).

References

- Hay, R. J., Macy, M. L. and Chen, T. R. (1989) *Nature* **339**:487.
- Bölske, G. (1988) *Zbl. Bakt. Hyg.* **A269**:331.
- McGaritty, G. J., Kotani, H. and Butler, G. H. (1993) Mycoplasmas and tissue culture cells in Mycoplasmas: *Molecular Biology and Pathogenesis*, ASM, Washington, 445-454.
- Wirth, M., Berthold, E., Grashoff, M., Pfützner, H., Schubert, U. and Hauser, H. (1994) *Cytotechnology* **16**:67.
- Kwok, S. (1990) Procedures to minimize PCR-product carry-over. In: *PCR Protocols: A Guide to Methods and Applications* (Innis, M. A., Gelfand, D. H., Sninsky, J. J., White, T. J., eds.), Academic Press, San Diego, 142-146.
- Drexler, H. G. et al. (1994) *In Vitro Cell. Dev. Biol.* **30A**:344.

Editor's note:

The Mycoplasma PCR ELISA simplifies mycoplasma detection. But once mycoplasmas are detected, the infected cultures must be treated or discarded, a solution you certainly will not want to apply on precious cell cultures. Several antibiotic preparations for curing cell cultures from mycoplasma infection are commercially available. Recently, the antibiotic combination BM-Cyclin was shown to be the only available antibiotic that, in a large panel of infected cell cultures, lead to a complete elimination of mycoplasmas; other antibiotics failed to remove mycoplasmas from 20% of the infected cultures tested (6). BM-Cyclin is an antibiotic combination containing a pleuromutilin derivative and a tetracycline derivative that are sequentially administered to the infected cell cultures to eliminate the mycoplasmas.

Product	Cat. No.	Size
Mycoplasma PCR ELISA*	1663 925	1 kit (96 reactions)

Also available	Cat. No.	Size
BM-Cyclin	799 050	treats 750–3000 ml
DAPI (4',6-Diamidine-2-phenylindole dihydrochloride)	236 276	10 mg
Mycoplasma Detection Kit (enzyme immunoassay)	1296 744	1 kit (25 tests)

See page 20 for local pricing.

*Purchase of this product is accompanied by a limited license to use it in the Polymerase Chain Reaction (PCR) process for molecular biology research in conjunction with a thermal cycler whose use in the automated performance of the PCR process is covered by the up-front license fee, either by payment to Perkin-Elmer or as purchased, i.e., an authorized thermal cycler.