PCR Mycoplasma – Test Kit I

Mycoplasma PCR Test Kit I
Product No. A9753

Description

PCR Mycoplasma Test Kit I is designed to detect the presence of mycoplasma contamination in biological materials, such as cultured cells. The detection by PCR requires only 1 to 5 femtograms of mycoplasma DNA. The primer set is specific to the highly conserved rRNA operon (i.e. the 16S rRNA coding region in the mycoplasma genome). The primer set allows for the detection of all in cell cultures occurring mycoplasma contaminants (*M. fermentans, M. arginini, M. orale, M. hyorhinis, M. salivarium, M. hominis*). In case of a contamination a PCR product of 267 bp in length is amplified. The primers are mycoplasma-specific; eukaryotic and other bacterial DNA is not amplified. Results are obtained within few hours.
This kit also provides internal control DNA, which should be added to all reaction in order to check the performance of the reaction. When running the PCR with the internal control DNA, a successful PCR is indicated by a 191 bp band on agarose gels.

Principle
rRNA gene sequences of prokaryotes, including mycoplasma, are well conserved, whereas, the lengths and sequences of the spacer region in the rRNA operon (for example the region between 16S and 23S gene) differ from species to species.
The detection procedure consists of 2 steps:
1. PCR: Amplification of a conserved and mycoplasma-specific 16S rRNA gene region (2 µl of sample volume per PCR)
2. Detection of the amplified fragment by agarose gel electrophoresis.

Components of the kit:	**Volume or form**	**A9753,0025 for 25 tests**
(RED) Mycoplasma Test Master Mix: primer sets, nucleotides, internal amplification control, and HotStart *Taq* DNA polymerase	lyophilized	A9753,0025A
(GREEN) Positive Control DNA: DNA-fragments of *Mycoplasma orale* genome (prepared by PCR, not infectious)	lyophilized	A9753,0025B
(WHITE) Tris Buffer (10 mM)	2 ml	A9753,0025C
(BLUE) Rehydration Buffer	1.3 ml	A9753,0025D

Storage: 2 – 8°C
After rehydration, keep all reagents on ice and store at -20°C. Avoid repeated freezing and thawing.

Materials required but not provided with the kit:
Agarose gel
DNA stain
PCR reaction tubes
Devices such as gel electrophoresis and documentation system, PCR thermocycler, pipetting equipment, tube centrifuge

Protocol

1. Sample Preparation

Sample Material/ Inhibiting Factors
Samples should be derived from cultures which are at 90-100 % confluence. PCR inhibiting substances may accumulate in the medium of older cultures. Penicillin or streptomycin in the culture media do not inhibit mycoplasma or affect test sensitivity. Cell culture supernatant is highly recommended to test for mycoplasma without further sample preparation. With average titers at 10^6 particles/ml and a maximum titer at 10^8 particles/ml sufficient mycoplasma DNA will be present in the supernatant to guarantee a sensitive PCR even for cell wall associated species.

Prepare the supernatant of cell cultures as follows:

1. Transfer 100 µl of supernatant from the test culture to a sterile micro centrifuge tube. The lid should be tightly sealed to prevent opening during heating.
2. Boil or incubate the sample supernatant at 95°C for 5 minutes.
3. Briefly centrifuge (5 seconds) the sample supernatant at approx. 13,000 rpm to pellet cellular debris before adding to the PCR mixture.
4. Use 2 µl of the supernatant of this centrifugation step for PCR. (Samples from this step may be stored at 2-8°C for up to 1 week.)

Other sample material
Cell pellets should not be tested directly, since debris will interfere with the PCR reaction. However, cell pellets as well as fetal calf serum, vaccines, cryo stocks and paraffin-embedded samples can be tested following DNA extraction. Use commercial kits for DNA extraction, such as Qiagen, QIAamp® DNA Mini Kit (No. 51 304).

2. PCR

Reconstitution of reagents

1. Centrifuge tubes with lyophilized components (spin 5 sec at maximum speed)
2. To Test Kit I Master Mix (RED cap) add 600 µl of Rehydration Buffer (BLUE cap)*

   *Note. For sample package (A9753,SAMPLE): to Test Kit I Master Mix add only 120 µl of Rehydration Buffer.
3. To Positive Control DNA (GREEN cap) add 300 µl of Tris Buffer (WHITE cap)
4. Incubate the reconstituted Master Mix and DNA (RED and GREEN caps) 5 min at RT. Then, vortex briefly and spin for 5 sec

After reconstitution, the reagents should be stored at -20 °C. Repeated freezing and thawing should be avoided. If you are testing only small numbers of samples at one time, store the reconstituted reagents Master Mix and DNA (RED and GREEN caps) in smaller aliquots.

Setting up samples and controls – Our "23:2 rule"
The test should be carried out with negative and positive controls and samples in duplicate. All reagents and samples must be equilibrated to 2-8 °C prior to use.

Pipette **23 µl** of the master mix into each reaction tube.
- For negative control add **2 µ**l of PCR grade Water (WHITE).
- Add **2 µl** of sample (as described above) to PCR reaction tube per sample being tested.
- For the positive control add **2 µ**l of Positive Control DNA (GREEN).

After pipetting the negative control, the tube must be sealed immediately before proceeding with the samples to minimize the risk of contamination carry-over. Also pipetting of the samples and sealing the tubes must be completed before proceeding with the positive control in order to avoid any cross contamination.

- Carefully mix the reactions, spin briefly and place the reaction tube into the thermocycler.
- Start PCR run.

PCR Run

Recommended thermocycling:

Initial heating*	2 minutes	94°C
39 cycles	30 sec	94°C
	30 sec	55°C
	30 sec	72°C

Cool down to 4 - 8°C

3. Gel Electrophoresis

Agarose Gel Run

1. 1.5 % standard agarose* gel, approx. 5 mm thick, with 5 mm-comb.
2. Load 5 µl of each PCR reaction, mixed with bromophenol blue loading buffer per lane.
   (In order to avoid interference with PCR products only bromophenol blue (BPB) in a low concentration should be used as a loading dye)
3. Stop electrophoresis after approx. 20 min @100 V (corresponding to approx. 2 cm run distance).

*e.g AppliChem Agarose low EEO (A2114) or Agarose Basic (A8963)

Gel Evaluation

Relevant amplicon sizes:

- Internal control: 191 bp (indicating a successfully performed PCR)
- *Mycoplasma spp.*: 265-278 bp (please see appendix)

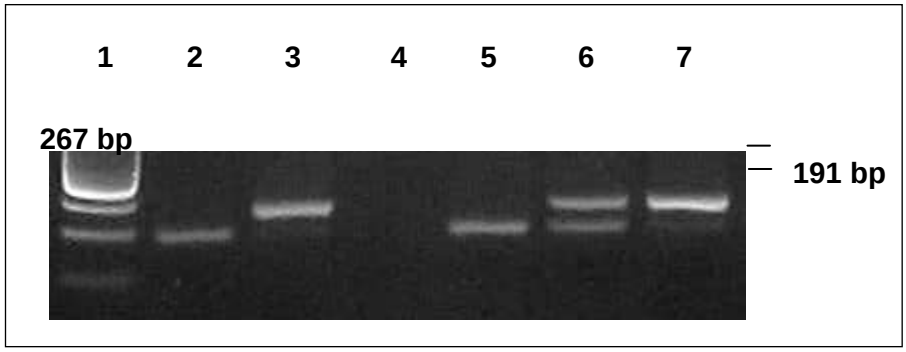
Mycoplasma-specific band at approx. 267 bp	**Internal control band at 191 bp**	**Interpretation of the sample** (sample DNA and internal control DNA)
positive	irrelevant*	Mycoplasma present in the sample
negative	negative#	PCR inhibition
negative	positive	No mycoplasma detectable in the sample

band at approx. 267 bp	**Internal control band at 191 bp**	**Controls**
positive (strong!)	negative or weak	*Positive control* (positive control DNA and internal control DNA)
negative	positive	*Negative control* (water and internal control DNA)

***Please note**: The internal control DNA band fades out with increased amounts of mycoplasma DNA-amplicons formed; especially if the mycoplasma DNA exceeds $5x10^6$ copies/ml. The initial concentration of positive control DNA is clearly higher than $5x10^6$ copies/ml. Consequently, the internal control is usually not or only weakly visible in the positive control reaction.

#In the absence of a mycoplasm-specific band a distinct 191 bp band should always appear. If this band is missing or clearly weaker than in the negative

Example gel



1. DNA marker (100 bp DNA ladder)
2. negative control
3. positive control
4. inhibited sample
5. negative sample
6. contaminated, positive sample
7. strongly contaminated positive sample

Figure: Typical agarose gel of PCR products from different controls and samples

Troubleshooting:

No amplification of control DNA may be due to the following reasons:

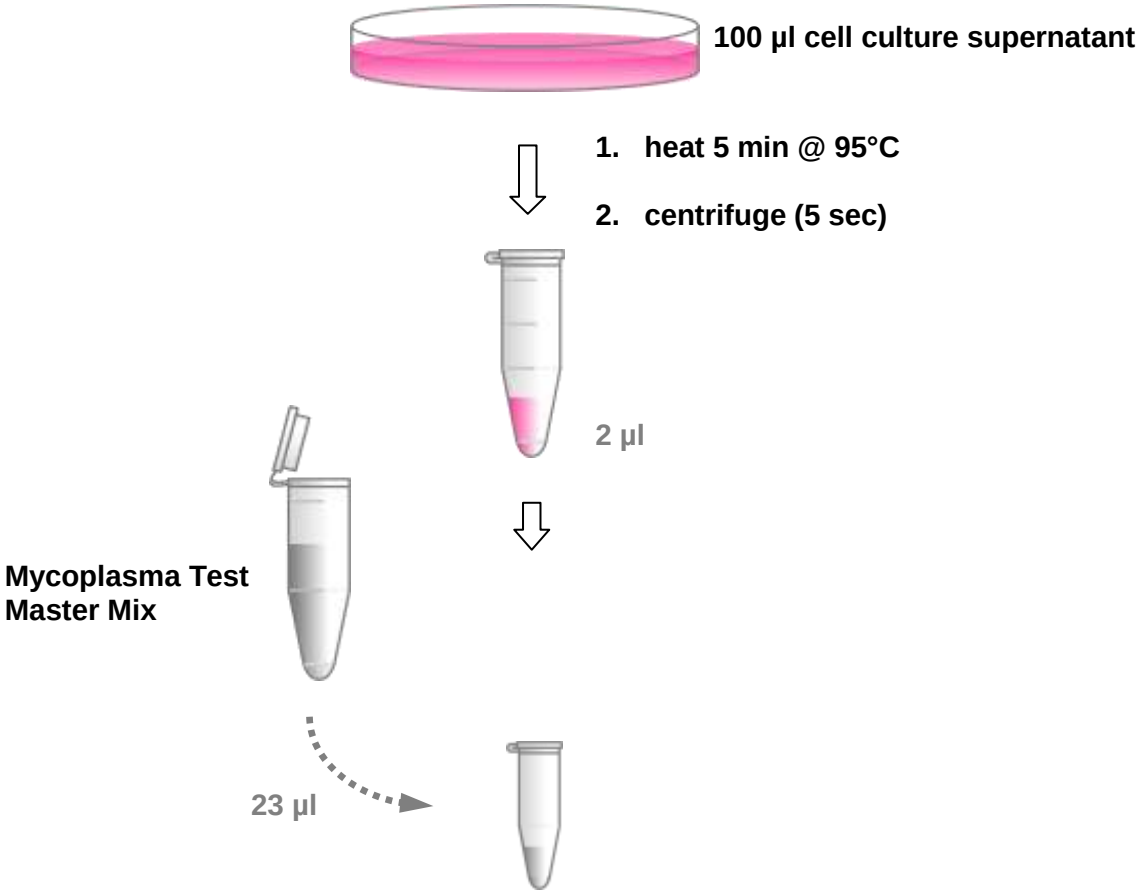
- control DNA tubes have not been spun down before rehydration
- programming mistake
- pipetting mistake

Rarely unspecific PCR products can be formed and become visible in the gel as faint, diffuse bands of different sizes (not 190 bp or 267 bp), but do not indicate positive results. These unspecific products are mainly caused by non-specific annealing due to overloading the PCR reaction with samples containing more than 100 µg/ml of DNA. Possible primer self-annealing produces another band of 80-90 bp in length, but does not affect the precision or results of the test.

In apparent inhibition of PCR by the sample (lower band intensity compared to negative control) a DNA extraction has to be performed and the sample will be tested again.

Before a re-run, please check thermocycler protocol and pipetting scheme!

Short Protocol



3. PCR (39 cycles)

5 µl

4. Agarose gel electrophoresis

Appendix

Analytical sensitivity
For all species tested 100% were detected at concentrations below 20 genomes/PCR.
Species and respective 100% cut-off
Acholeplasma laidlawii: < 20, *Mycoplasma fermentans:* < 10, *Mycoplasma synoviae:* < 20, *Mycoplasma pneumonia:* < 20, *Spiroplasma citri:* < 20

Cross-reactivity
No cross-reactivity has been observed with eukaryotic DNA. Unspecific PCR products may rarely be formed and become visible in the gel as faint, diffuse bands of different sizes due to overloading the PCR. The kit is also not detecting any of the phylogenetically related microorganisms Clostridium, Lactobacillus and Streptococcus. Likewise, the water-born germ Burgholderia is not detected. The following species have been tested with PCR Mycoplasma–Test Kit I (A9753).

Detected species with respective amplicon sizes [bp]

Acholeplasma laidlawii 273
Mycoplasma agalactica 267
Mycoplasma arginini 267
Mycoplasma arthritidis 267
Mycoplasma bovigenitalium 267
Mycoplasma bovis 267
Mycoplasma caprine 267
Mycoplasma cloacale 266
Mycoplasma falconis 268
Mycoplasma faucium 265
Mycoplasma fermentans 267
Mycoplasma genitalium 273
Mycoplasma hominis 266
Mycoplasma hyorhinis 268
Mycoplasma hyosynoviae 265
Mycoplasma opalescens 266
Mycoplasma orale 266
Mycoplasma penetrans 274
Mycoplasma pirum 274
Mycoplasma pneumoniae 273
Mycoplasma pulmonis 268
Mycoplasma salivarium 266
Mycoplasma spermatophilum 267
Mycoplasma synoviae 266
Mycoplasma timone 266
Spiroplasma citri 268

A large number of Mycoplasma sequences have been published. The primers of the kit were aligned with the NCBI data base and inspected for homologies within the target region of the 16S rRNA. The following list shows mycoplasma species with relevant sequence homologies and highest presumption of a positive PCR result.
M. agassizii, M. canadense, M. columborale, M. gallopavonis, M. iners, M. sphenisci, M. alkalescens, M. capricolum, M. cynos, M. gateae, M. lagogenitalium, M. spumans, M. anseris, M. caviae, M. edwardii, M. glycophilum, M. lipofaciens, M. sualvi, M. bovirhinis, M. citelli, M. equirhinis, M. gypis, M. lipophilum, M. subdolum, M. buccale, M. collis, M. felifaucium, M. hyopharyngis, M. meleagridis, M. testudineum, M. buteonis, M. columbinasale, M. gallinaceum, M. iguana, M. moatsii, M. turnidae, M. californicum, M. columbinum, M. gallinarum, M. indiense, M. simbae, M. verecundum

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