PCR Mycoplasma Test Kit II

Kit for Mycoplasma detection by conventional PCR
Product code A8994

Description

PCR Mycoplasma Test Kit II 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 (corresponding to only 2-5 mycoplasma per sample volume). 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 frequently occurring contaminants (*M. fermentans, M. arginini, M. orale, M. hyorhinis, M. salivarium, M. hominis*). In addition, more species are detected, such as *M. pneumoniae, Acholeplasma laidlawii, M. synoviae* and Ureaplasma species. In case of a contamination a PCR product of 270 bp in length will be 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 can be added to the reaction in order to check the performance of the reaction. When running the PCR with the internal control DNA, a successfull PCR is indicated by a 191 bp band on agarose gels.

PCR Mycoplasma Test Kit II meets the criteria of the European Pharmacopeia (Ph. Eur.), section 2.6.7.

Principle
rRNA gene sequences of prokaryotes, including mycoplasmas, 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 utilizing the PCR process with this primer set consists of:
1. Amplification of a conserved and mycoplasma-specific 16S rRNA gene region.
2. Detection of the amplified fragment by agarose gel electrophoresis.

Components of the kit	**A8994,0025 for 25 tests**	**A8994,0050 for 50 tests**	**A8994,0100 for100 tests**
(RED) Primer/Nucleotide Mix: lyophilized primer set and nucleotides dATP, dCTP, dGTP, dUTP, aliquoted for 25 reactions	A8994,0025B	2x A8994,0025B	4x A8994,0025B
(BLUE) PCR 10X Reaction Buffer: 500 µl buffer solution	A8994,0025A	2x A8994,0025A	4x A8994,0025A
(GREEN) Positive Control DNA: lyophilized DNA-fragments of *Mycoplasma orale* genome (prepared by PCR, not infectious)	A8994,0025C	2x A8994,0025C	4x A8994,0025C
(YELLOW) Internal Control: lyophilized DNA Plasmid-DNA (not infectious)	A8994,0025I	2x A8994,0025I	4x A8994,0025I
(WHITE) PCR grade Water: 2 ml deionized, DNA-free water for rehydration of the lyophilized kit components and PCR mixtures	A8994,0025H	2x A8994,0025H	4x A8994,0025H

Storage: 2 – 8°C (After rehydration, keep all reagents on ice and store below -20°C)

Reagents required but not provided with the kit
Taq DNA polymerase, we recommend SuperHo*t Taq* DNA Polymerase (AppliChem Product code A5231)

Protocol

1. Sample Preparation

Sample Material/ Inhibiting Factors
Samples should be derived from cultures which are at 90-100 % confluence. In the medium of older cultures PCR inhibiting substances may accumulate. For these sample materials a DNA extraction is strictly recommended prior to testing.
Penicillin or streptomycin in the culture media do not inhibit mycoplasma or affect test sensitivity. Only cell culture supernatant should be applied to test for mycoplasma.
Cell pellets should not be tested, since debris will interfere with the PCR reaction. With average titers at 10^6 and a maximum titer at 10^8 sufficient amounts of mycoplasma DNA is present in the supernatant to guarantee a sensitive PCR. However, cell pellets as well as Fetal Calf Serum, vaccines, and paraffin-embedded samples can be tested following DNA extraction.

Prepare the supernatant of cell cultures (or other test material) 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.)

2. PCR

Reconstitution of reagents

1. Centrifuge tubes with lyophilized components (spin 5 sec at maximum speed)
2. Add appropriate amount of deionized, DNA-free water (WHITE):
   - Primer/Nucleotide Mix (per portion of 25 reactions, RED): 65 µl
   - Positive Control DNA (GREEN): 300 µl
   - Internal Control DNA (YELLOW): 300 µl
3. Incubate for 5 minutes at room temperature
4. Vortex and centrifuge again

After rehydration, keep all reagents on ice and store below -20°C. To avoid repeated freezing and thawing Primer/Nucleotide Mix and controls should be aliquoted after reconstitution. It is also possible to store aliquots of the mastermix at -20°C.

PCR master mix
When setting up reactions, calculations should also include positive and negative controls. Total volume per reaction is 25 µl.

Pipetting schemes:

	for 1 reaction	for 5 reactions	for 25 reactions
PCR grade Water (WHITE cap)**	15.3 µl	76.5 µl	382.5 µl
10X Reaction Buffer (BLUE cap)	2.5 µl	12.5 µl	62.5 µl
Primer/Nucleotide Mix (RED cap)*	2.5 µl	12.5 µl	62.5 µl
Internal Control (YELLOW cap)	2.5 µl	12.5 µl	62.5 µl
Polymerase (5 U/µl)**	0.2 µl	1.0 µl	5.0 µl

* Content of one red-capped vial totals 25 reactions.
** For other polymerase concentrations the amount of water needs to be adjusted.

Pipette 23 µl of the mastermix into each reaction tube.

- For negative control add 2 µl 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 µl of Positive Control DNA (GREEN).

After pipetting the negative control, the tube must be sealed before proceeding with the samples. Also pipetting of the samples and sealing the tubes must be completed before proceeding with the positive control in order to avoid 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		

* Initial incubation time depends on the *Taq* DNA polymerase used. Some hot start enzymes need to be activated at 94°C for more than 2 minutes. Please see polymerase data sheet for activation time.

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
If internal control DNA was used, a distinct 191 bp band should appear in every lane indicating a successfully performed PCR. This band may fade out with increased amounts of amplicons formed, caused by mycoplasma DNA loads of > $5 \cdot 10^6$ copies/ml. The initial concentration of positive control DNA exceeds $5 \cdot 10^6$ copies/ml in order to account for DNA loss resulting from repeated freezing and thawing.

Relevant amplicon sizes:
- Internal control: 191 bp
- *Mycoplasma spp.*: 265-278 bp (please see appendix)

Results of successfully performed PCR

negative control :	band at 191 bp
positive control:	band at 267 bp , possibly an additional band at 191 bp

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

- activity of polymerase is insufficient
  The concentration of *Taq* DNA polymerase can be raised up to 2.5 U/reaction. Please note: this results in the complete change of the pipetting scheme.
- control DNA tubes have not been spun down before rehydration
- programming mistake
- pipetting mistake

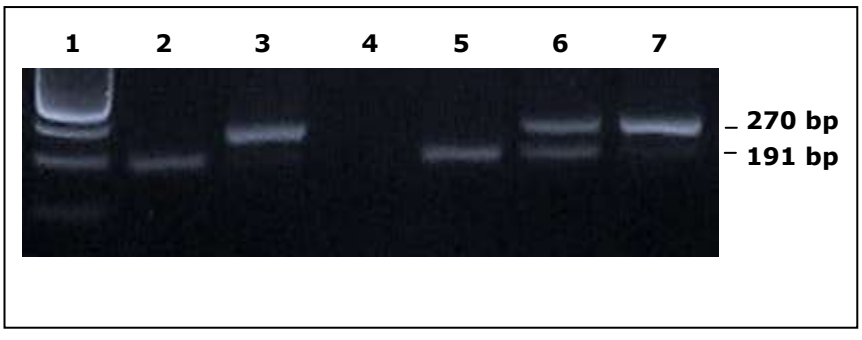
Before a re-run, please check thermocycler protocol and pipetting scheme!

Interpretation of possible band patterns

band only at 191 bp:	negative sample. But caution: if the signal is significant weaker than in the internal control the PCR is partly inhibited!
band at ~270 bp and at 191 bp:	mycoplasma-positive sample with weak contamination
strong band at 270 bp:	mycoplasma-positive sample, highly contaminated
no band:	PCR inhibition or insufficient activity of polymerase

With PCR Mycoplasma Test Kit II designed for high sensitivity and therefore prone to nonspecific annealing, bands of various length that are less intensive can be produced, but do not indicate positive results. Possible primer self-annealing produces another band of 80-90 bp in length, but also does not affect the precision or results of the test.

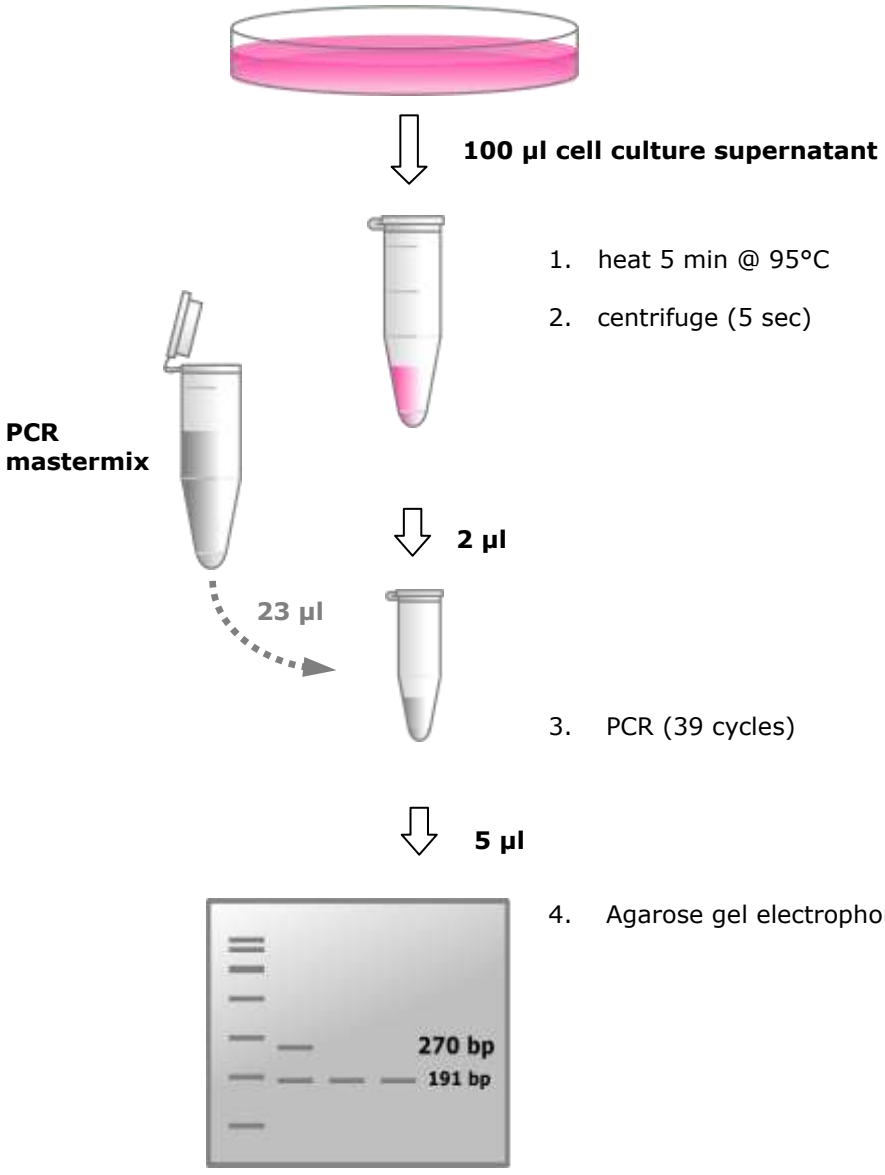
If the PCR of a sample is inhibited, PCR inhibitors can easily be removed from the sample by performing a DNA extraction with a commercially available kit.



Agarose gel of PCR products from different controls and specimen.

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.

Short Protocol



Appendix

Species and respective amplicon size (bp)

Mycoplasma orale[1,2] (266)
Mycoplasma pneumoniae[2] (273)
Acholeplasma laidlawii[2] (273)
Mycoplasma hyorhinis[2] (268)
Mycoplasma synoviae[2] (266)
Mycoplasma penetrans (274)
Mycoplasma pirum (274)
Mycoplasma fermentans (267)
Ureaplasma urealyticum (273)
Mycoplasma pulmonis (268)
Mycoplasma falconis (268)
Mycoplasma arthritidis (267)
Mycoplasma arginini (267)
Mycoplasma spermatophilum (267)
Mycoplasma opalescens (266)
Mycoplasma primatum (267)
Mycoplasma maculosum (267)
Mycoplasma bovis (267)
Mycoplasma cloacale (266)
Mycoplasma hyosynoviae (265)
Mycoplasma salivarium (266)
Mycoplasma faucium (265)
Mycoplasma hominis (266)
Mycoplasma genitalium (273)
Mycoplasma bovigenitalium (267)
Mycoplasma sp. ovine/caprine (267)
Mycoplasma agalactica (267)
Mycoplasma timone (266)

1 provided as positive control DNA
2 Mycoplasma test strain according to European Pharmacopoeia, Suppl. 2000, 2.6.7.

Version MM20131223