# qPCR Mycoplasma Test Kit

*Real time/qPCR test kit for mycoplasma detection*
**Product code A9019**

## Description

The Mycoplasma PCR Detection Kit is based on a 5-Nuclease probe assay for the quantitative polymerase chain reaction (PCR), which is established as the method of choice for highest sensitivity in the detection of *Mycoplasma* and *Acholeplasma* contamination in cell cultures and other cell culture derived biologicals.
The primer/probe system detects the highly conserved 23S rRNA operon coding region of the mycoplasma genome. The kit is highly specific and does not detect eukaryotic DNA. The detection range includes most mycoplasma species identified as cell culture contaminants (see Table 2). The kit contains the nucleotide dUTP instead of dTTP and is, therefore, suitable for UNG pretreatment.

The kit contains an optional Inhibition Control (Internal Control DNA). The Internal Control DNA can be added to the master mix to detect incomplete PCR or directly to a sample prior processing the complete DNA isolation and purification process. An internal control probe is already included in the Reaction Mix, which also contains the *Taq* DNA Polymerase enzyme. This probe emits fluorescent light with amplification of the Internal Control DNA confirming mycoplasma-negative, but successfully performed test reactions.

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| **Components of the kit:** | **A9019,0025 for 25 Tests** | **A9019,0100 for 100 Tests** |
|---|---|---|
| **(RED) Reaction Mix:** lyophilized primers, probe, *Taq* DNA Polymerase and deoxynucleotide triphosphates dATP, dCTP, dGT and dUTP, aliquoted for 25 reactions | A9019,0025A | 4x A9019,0025A |
| **(BLUE) Rehydration Buffer** (1.8 ml each) | A9019,0025B | 4x A9019,0025B |
| **(GREEN) Positive Control DNA:** non-infectious, lyophilized plasmid DNA | A9019,0025C | 4x A9019,0025C |
| **(YELLOW) Internal Control DNA:** non-infectious, lyophilized plasmid DNA | A9019,0025D | 4x A9019,0025D |
| **(WHITE) PCR Grade Water:** water for dissolving the components and setting up the master mix | A9019,0025E | 4x A9019,0025E |

**Shipment:** Room temp.
**Storage:** Kit components should be stored at 2–8 ºC.
After reconstitution of the Reaction Mix, the Positive Control and the Internal Control shall be stored at –20ºC. Avoid repeated freezing and thawing. For repeated testing of low sample numbers, Reaction Mix and controls should be aliquoted after reconstitution. It is also possible to store aliquots of the Reaction Mix at -20°C.

**Equipment and Reagents Required But not Provided with the Kit:**
PCR reaction tubes and 1.5 ml reaction tubes for the master mix (DNA- and RNA-free!), microcentrifuge, micropipettes, and filter tips.

**qPCR machine:**
This qPCR Mycoplasma Test Kit is suited for use in combination with different instruments:

| | | |
|---|---|---|
| LightCycler® 2.0, 480 | iCycler iQ® | Chromo 4 |
| Rotor-Gene™ 3000, 6000 | iQ™5 | MX3005P® |
| ABI Prism® 7500 | Opticon 2 | MX4000® |

*Precautions and Disclaimer*

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

## Protocol

### 1. Preparation of Sample Material

Cell lines should be pre-cultured in the absence of mycoplasma active antibiotics for several days to maximize test sensitivity. Samples should be derived from cultures, which are at 90–100 % confluence. **PCR inhibiting substances may accumulate in the medium of older cultures. For a sample from an older culture, a DNA extraction is strictly recommended prior testing.**

Penicillin and streptomycin in the culture medium do not inhibit mycoplasma nor affect test sensitivity. **Cell culture supernatant is preferred to test for mycoplasma.** Cell pellets should only be tested after suitable DNA extraction, since debris will interfere with the PCR reaction. With average titers of $10^6$ particles/ml and a maximum titer of $10^8$ particles/ml, sufficient mycoplasma is found in the supernatant to guarantee a sensitive PCR. However, other materials that can be tested are fetal calf serum, vaccines, and paraffin-embedded samples following DNA extraction. If necessary, templates for PCR analysis are prepared by DNA extraction. Please make sure to remove any alcohol containing wash buffer from the preparation to avoid co-elution of alcohol and sample material. Any remaining alcohol may inhibit the PCR. 2 µl of the extract can be used directly as PCR template.

To avoid false positive results, the use of the PCR grade water delivered with the kit, aerosol-preventive filter tips, and gloves is recommended.

**Sample material may be prepared by one of the following methods:**

## Heat-Inactivation of the Sample Material

Sample material derived from cell cultures contains DNAses which can degrade the mycoplasma DNA even at low temperatures. Therefore, we recommend heat-inactivation of the material if DNA extraction cannot be performed immediately after sampling. The templates for the PCR analysis are prepared by direct heating of the cell culture supernatant or the biological sample material:

1. Transfer 500 µl of supernatant (or cell culture material with max. $10^6$ cells) from the test culture to a sterile tube. The lid should be tightly sealed to prevent opening during heating.

2. Boil or incubate the sample supernatant at 95ºC for 10 minutes.

3. Briefly centrifuge (5 seconds at 13,000 rpm) the sample supernatant to pellet cellular debris. The template DNA is stable at 2–8ºC for 1 week, and for several months at -20°C. Repeated freezing and thawing should be avoided.

2 µl of the supernatant is used in the PCR. Alternatively, the DNA can be purified with a commercial extraction kit.

## Enrichment of Mycoplasma by Centrifugation

1. Transfer 1 ml of supernatant from the test culture to a sterile amplification tube. The lid should be tightly sealed to prevent opening during heating.

2. Centrifuge the supernatant for 15 minutes at 10,000 x *g* to sediment mycoplasma particles. Alternatively: centrifuge the supernatant 6 minutes at 13,000 x *g*.

3. Remove the supernatant and suspend the pellet in 50 µl of buffer (10 mM Tris, pH 8.4).

4. The sample should be vortexed and finally heated up to 95ºC for 10 minutes.

The extracts can be stored at –20ºC for a period of one year. Repeated freezing and thawing, or storage in the refrigerator for longer than 12 months should be avoided. The sample should not contain more than 100 µg/ml DNA.

## 2. PCR

**Preparation Instructions**
Reconstitution of the Reagents

1. Centrifuge tubes with lyophilized components (5 seconds at maximum speed).
2. Add 600 µl of Rehydration Buffer (BLUE) to the Reaction Mix (RED).
3. Add 300 µl of deionized, DNA-free water (WHITE) to each Positive Control (GREEN) and Internal Control (YELLOW).
4. Incubate for 5 minutes at room temperature.
5. Mix and centrifuge again.

**The PCR Master mix**

The total volume per reaction is 25 µl. When setting up reactions, calculations should also include positive and negative controls.
**The total duration from master mix preparation to PCR cycling must not exceed 45 minutes to avoid a decrease in the fluorescence signal!**

| | **1 reaction** | **25 reactions** |
|---|---|---|
| Reaction Mix | 22 µl | 550µl |
| Internal Control DNA | 1.0 µl | 25.0 µl |
| + template DNA: Sample, Negative control or Positive control | 2.0 µl | |

Pipette 23 µl of master mix (22 µl of Reaction Mix and 1 µl of Internal Control DNA) into each PCR reaction tube and mix gently.
Add 2 µl of prepared sample to PCR reaction tube per sample being tested. After pipetting the negative control (2 µl of water or negative control of DNA extraction/ reaction), 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 (2 µl/reaction) in order to avoid cross contamination.

**Programming of the Cycler**

For all used vials set detectors for Mycoplasma Target Probe to FAM™ and for the Internal Control Probe to HEX™. No quencher must be specified. Inactivate available HEX™ Reference functions. Fluorescence should be measured during extension. **Please see APPENDIX for more details!**

| Program Step 1: | Pre-incubation |
|---|---|
| Setting | Hold |
| Temperature | 95°C |
| Incubation time | 3:00 min |

| Program Step 2: | Amplification |
|---|---|
| Cycles | 45 |
| Denaturing | 95°C for 30 sec |
| Annealing & Reading | 55°C for 30 sec |
| Extension | 60°C for 45 sec |

## 3. Results

A successfully performed PCR without inhibition is indicated by an increasing fluorescence signal in the internal control channel (HEX™), provided the Internal Control was added to the master mix. The internal control can be detected with an orange filter (530–560 nm for HEX™). The presence of mycoplasma DNA in the sample is indicated by an increasing fluorescence signal at 520 nm (FAM™) and is usually detected with a green filter (470–510 nm).

Mycoplasma DNA and Internal Control DNA are competitors in PCR. Because of the very low concentration of Internal Control in the PCR mix, the signal strength in this channel is reduced with the increasing mycoplasma DNA loads in the sample. False-negative results, e.g. due to inhibition of the reaction by the sample matrix, can be detected individually for each sample as these reactions do not show any fluorescence signal.

**Figure**

A) Detection Chanel 1: Mycoplasma Detection
The FAM™ signal indicates amplification of Mycoplasma-specific genes.

B) Detection Chanel 2: Internal Run Control
The HEX™ signal indicates amplification of the internal control. If the $C_t$-value of the internal control within a Mycoplasma-negative sample is higher than the $C_t$-value of the pure internal control sample the PCR reaction is at least partly inhibited and the reaction should be repeated with extracted sample DNA.

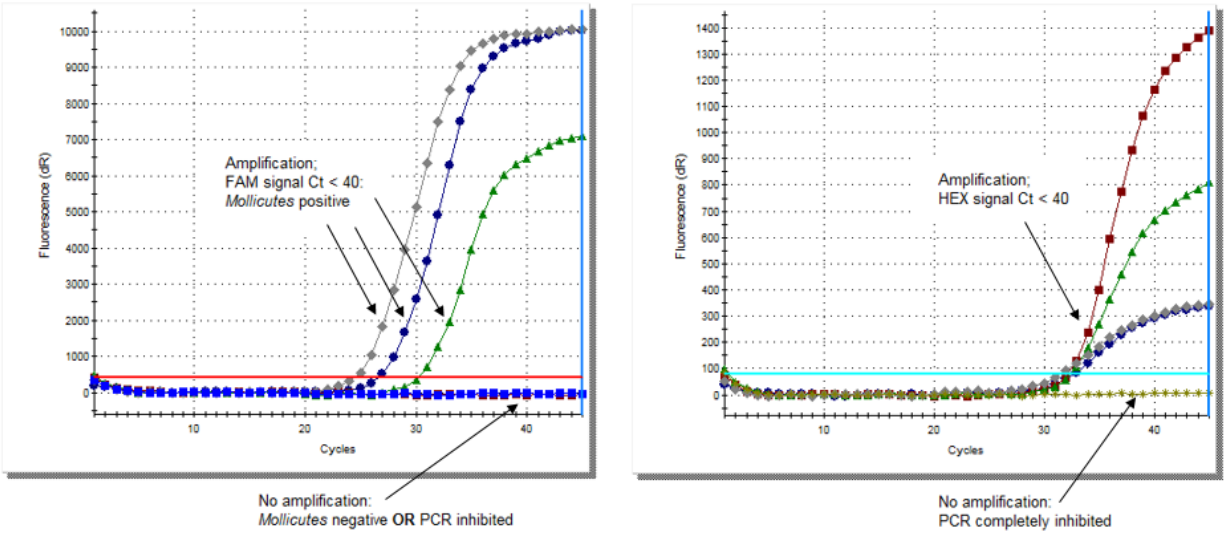
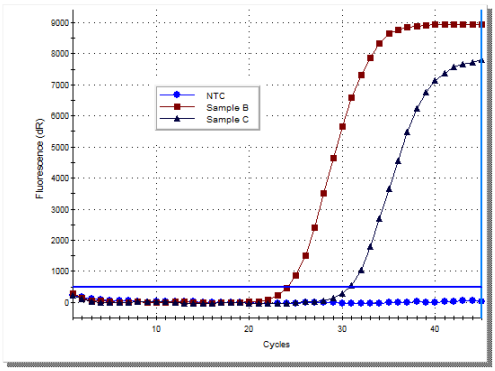



**Table 1:** Interpretation of SAMPLE-PCR Results

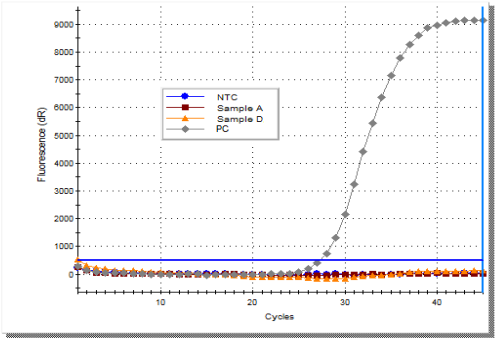
| **Mycoplasma FAM™ channel** | **Detection Internal Control HEX™ channel** | **Interpretation** |
|---|---|---|
| Positive (Ct < 40) | Irrelevant | Mycoplasma (*Mollicutes*) positive |
| Negative | Negative | PCR inhibition |
| Negative | Positive (Ct < 40), but Ct > Ct (internal control) | PCR inhibition (partly) |
| Negative | Positive | Mycoplasma (*Mollicutes*) negative |
| Borderline (Ct > 40) | Positive (Ct < 40) | Result not valid, repeat process including DNA extraction |
| Borderline | Negative | PCR inhibition |

**Figure 2:** Interpretation of qPCR Results: Practice

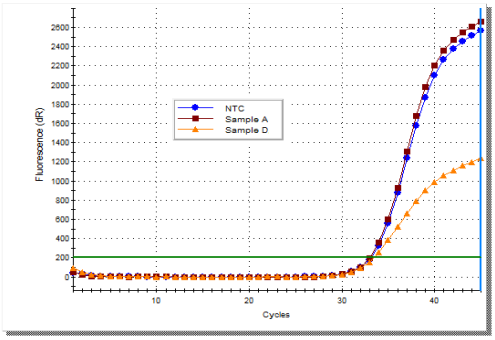
A) FAM™ channel:
Samples with $C_t$-values of 25 (B, red) and 31(C, black) in comparison with negative control (NTC, blue). Both samples are clearly contaminated with mycoplasma DNA, interpretation of the internal run channel is not required.




B) FAM™ channel:
Samples with $C_t$-values > 45 (A, red and D, orange) compared to negative control (NTC, blue) and positive control (PC, grey). In both samples, no amplification of mycoplasma DNA was detected. As a consequence, the samples do either not contain any mycoplasma DNA – or the PCR-reaction was inhibited. To interpret the results the amplification of the internal control DNA needs to be checked!




C) HEX™ channel:
For interpretation of the FAM™-negative samples A and D, the HEX™ channel results need to be analysed. While the internal control curve of sample A (red) is comparable to the negative control (NTC)(blue), the amplification curve of sample D (orange) runs lower. This result refers to a slight inhibition of the PCR reaction in sample D and therefore this sample has to be analyzed again. A DNA extraction is highly recommended to remove potential PCR inhibitors from the sample. Internal control DNA needs to be checked!




**Troubleshooting**

- **No amplification of the control DNA** may be due to the following:
  - ~ programming error
  - ~ pipetting error
- Before re-running the controls, check thermocycler program and pipetting scheme.
- If the PCR of a sample is inhibited: **PCR inhibitors can be easily removed from the sample by performing a DNA extraction** using e.g. Geno/miracle DNA Isolation Spin-Kit (AppliChem product code A5179).

# APPENDIX

## 1. Species specificity

The following species are included:

| | | |
|---|---|---|
| *Acholeplasma laidlawii* | *Mycoplasma conjunctivae* | *Mycoplasma iguanae* |
| *Mycoplasma agalactiae* | *Mycoplasma crocodyli* | *Mycoplasma imitans* |
| *Mycoplasma alligatoris* | *Mycoplasma fermentans* | *Mycoplasma mobile* |
| *Mycoplasma alvi* | *Mycoplasma flocculare* | *Mycoplasma orale* |
| *Mycoplasma amphoriforme* | *Mycoplasma gallisepticum* | *Mycoplasma ovipneumoniae* |
| *Mycoplasma anatis* | *Mycoplasma genitalium* | *Mycoplasma pneumoniae* |
| *Mycoplasma arginini* | *Mycoplasma hominis* | *Mycoplasma pulmonis* |
| *Mycoplasma arthritidis* | *Mycoplasma hyopharyngis* | *Mycoplasma synoviae* |
| *Mycoplasma bovis* | *Mycoplasma hyopneumoniae* | *Mycoplasma testudineum* |
| *Mycoplasma canis* | *Mycoplasma hyorhinis* | *Mycoplasma testudinis* |
| *Mycoplasma columbinum* | *Mycoplasma hyosynoviae* | |

## 2. Programming and Data Recording

**LightCycler® 2.0 exclusively**

Program 1: Pre-incubation

| | |
|---|---|
| Cycles | 1 |
| Analysis Mode | None |
| **Temperature Targets** | **Segment 1** |
| Target Temperature [°C] | 95 |
| Incubation time [min] | 180 |
| Temperature Transition Rate [°C/s] | 20.0 |
| Secondary Target Temperature [°C] | 0 |
| Step Size [°C] | 0.0 |
| Step Delay [Cycles] | 0 |
| Acquisition Mode | None |

Important for LightCycler® 2.0:
Please check the correct settings for the "seek temperature" of at least 90 °C.

Program 2: Amplification

| | | | |
|---|---|---|---|
| Cycles | 45 | | |
| Analysis Mode | Quantification | | |
| **Temperature Targets** | **Segment 1** | **Segment 2** | **Segment 3** |
| Target Temperature [°C] | 95 | 55 | 60 |
| Incubation time [s] | 30 | 30 | 45 |
| Temperature Transition Rate [°C/s] | 20.0 | 20.0 | 20.0 |
| Secondary Target Temperature [°C] | 0 | 0 | 0 |
| Step Size [°C] | 0 | 0 | 0 |
| Step Delay [Cycles] | 0 | 0 | 0 |
| Acquisition Mode | None | None | Single |

Program 3: Cooling

| | |
|---|---|
| Cycles | 1 |
| Analysis Mode | None |
| **Temperature Targets** | **Segment 1** |
| Target Temperature [°C] | 40 |
| Incubation time [min] | 30 |
| Temperature Transition Rate [°C/s] | 20.0 |
| Secondary Target Temperature [°C] | 0 |
| Step Size [°C] | 0 |
| Step Delay [Cycles] | 0 |
| Acquisition Mode | None |

Analysis:

- Select the fluorescence channels Channel 1 (520 nm) and 2 (555 nm)
- Click on "Quantification" to generate the amplification plots and the specific $C_t$-values
- The threshold will be generated automatically.

## RotorGene® 6000 (5-plex)

Please check the correct settings for the filter combination:

| Target | Mollicutes | Internal Control |
|---|---|---|
| filter | green | yellow |
| wavelength | 470—510 nm | 530-560 nm |

Program 1: Pre-incubation

| | |
|---|---|
| Setting | Hold |
| Hold Temperature | 95 °C |
| Hold Time | 3 min 0 sec |

Program 2: Amplification

| | |
|---|---|
| Setting | Cycling |
| Cycles | 45 |
| Denaturation | 95°C for 30 sec |
| Annealing | 55°C for 30 sec |
| Elongation | 60°C for 45 sec → acquiring to Cycling A (green and yellow) |
| Gain setting | automatic (Auto-Gain) |
| Slope Correct | activated |
| Ignore First | deactivated |

Analysis:

- Open the menu "Analysis"
- Select "Quantitation"
- Check the required filter set (green and yellow) according to the following table and start data analysis by double click.
- The following windows will appear:

Quantitation Analysis - Cycling A (green / yellow)
Quant. Results - Cycling A (green / yellow)
Standard Curve - Cycling A (green / yellow)

- In window "Quantitation Analysis", select first "Linear Scale" and then "Slope Correct":
- Threshold setup (not applicable if a standard curve was carried with the samples and auto threshold was selected)
- In window "CT Calculation" set the threshold value to 0-1
- Pull the threshold line into the graph. Adapt the threshold line to the initial linear section of the positive control reaction.
- The $C_t$-values can be taken from the window "Quant. Results".
- Samples showing no $C_t$-value can be considered as negative.

## ABI Prism® 7500

Please check the correct settings for the filter combination:

| Target | Mollicutes | Internal Control |
|---|---|---|
| filter | FAM™ | HEX™ |
| wavelength | 470—510 nm | 530-560nm |
| quencher | none | none |

Important: The HEX™ Reference needs to be disabled. Activate both detectors for each well. Measurement of fluorescence during extension.

| Program 1: Pre-incubation | |
|---|---|
| Setting | Hold |
| Temperature | 95°C |
| Incubation time | 3 min |
| Program 2: Amplification | |
| Cycles | 45 |
| Setting | Cycle |
| Denaturing | 95°C for 30 sec |
| Annealing | 55°C for 30 sec |
| Extension | 60°C for 45 sec |

Analysis:

- Enter the following basic settings at the right task bar:

Data: Delta RN vs. Cycle
Detector: FAM™ and HEX™
Line Colour: Well Colour

- Open a new window for the graph settings by clicking the right mouse button.

Select the following settings and confirm with ok:
- ~ Real Time Settings: Linear
- ~ Y-Axis Post Run Settings: Linear and Auto Scale
- ~ X-Axis Post Run Settings: Auto Scale
- ~ Display Options: 2

- Initiate the calculation of the $C_t$-values and the graph generation by clicking on "Analyse" within the report window.
- Pull the threshold line into the graph. Adapt the threshold line to the initial linear section of the positive control reaction.
- Samples showing no $C_t$-value can be considered as negative.

## Mx3005P®

- Go to the setup menu, click on "Plate Setup", check all positions which apply
- Click on "Collect Fluorescence Data" and check FAM™ and HEX™
- Corresponding to the basic settings the „Reference Dye" function should be deactivated
- Specify the type of sample (no template control or positive control, sample, standard) at "well type"
- Edit the temperature profile at "Thermal Profile Design":

Segment 1:

| | |
|---|---|
| Setting | 1 cycle |
| Temperature | 95°C |
| Incubation time | 3 min |

Segment 2:

| | |
|---|---|
| Setting | 45 cycles |
| Denaturing | 30 sec 95 °C |
| Annealing | 30 sec 55 °C |
| Extension | 45 sec 60 °C |

- Select "Run" at menu "Run Status" and start the cycler by pushing "Start"

Analysis:

- In the window "Analysis" tab on "Analysis Selection / Setup" to analyze the marked positions
- Ensure that in window "Algorithm Enhancement" all options are activated:

Amplification-based threshold
Adaptive baseline
Moving average

- Click on "Results" and "Amplification Plots" for an automatic threshold
- Read the $C_t$-values at "Text Report"

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