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Product Information

LookOut® Mycoplasma qPCR Detection Kit

Catalog Number **MP0040**
Storage Temperature 2–8 °C

TECHNICAL BULLETIN

Product Description

The LookOut® Mycoplasma PCR Detection Kit utilizes the 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 16S rRNA operon coding region of the mycoplasma genome. The kit is highly specific and does not detect eukaryotic DNA. The detection spectrum 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 Internal Control DNA (an optional Inhibition Control). The Internal Control DNA can be added to the MasterMix to detect incomplete PCR or directly to a sample prior to complete DNA isolation and the purification process. An internal control probe is already included in the Primer/Probe/ Nucleotide Mix. This probe emits fluorescent light with amplification of the Internal Control DNA confirming mycoplasma-negative, but successfully performed test reactions.

This kit has been tested and optimized for use with JumpStart™ *Taq* DNA Polymerase, Catalog Number D9307. It is **highly recommended** that this product be used with the kit. Use of other *Taq* polymerase products may require reaction optimization to achieve proper results.

Components

- Primer/Probe/Nucleotide Mix 1 each
  Catalog Number M1449
  lyophilized primers, probe, and deoxynucleotide triphosphates dATP, dCTP, dGTP and dUTP, aliquoted for 25 reactions

- Rehydration Buffer (1.8 ml) 1 each
  Catalog Number R9282

- Positive Control DNA 1 each
  Catalog Number D1696
  non-infectious, lyophilized DNA-fragments of *M. orale*, *A. laidlawii*, and *M. pneumoniae* prepared by PCR

- Internal Control DNA 1 each
  Catalog Number D1571
  non-infectious, lyophilized Plasmid DNA

- PCR Grade Water 1 each
  Catalog Number W0645
  water for resolving the components and setting up the master mix

Equipment and Reagents Required but Not Provided

- qPCR machine
- Corresponding PCR reaction tubes
- Microcentrifuge, micropipettes, and filtered tips (1–1,000 µl)
- JumpStart *Taq* DNA Polymerase, Catalog Number D9307

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.

Preparation Instructions

Reconstitution of the Reagents

1. Centrifuge tubes with lyophilized components (5 seconds at maximum speed)

2. Add 365 µl of Rehydration Buffer to the Primer/Probe/Nucleotide Mix

3. Add 300 µl of PCR Grade Water to each Positive Control and Internal Control

4. Incubate for 10 minutes at room temperature

5. Vortex and centrifuge again

Storage/Stability
Kit components are stable during shipping at ambient temperature. Upon receipt, store at 2–8 °C.

After reconstitution of the Primer/Probe/Nucleotide Mix, the Positive Control, and the Internal Control, store at –20 °C and avoid repeated freezing and thawing. For repeated testing of low sample numbers, Primer/Probe/ Nucleotide Mix and controls should be aliquoted after reconstitution.

Procedure
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 to testing. The GenElute™ Blood Genomic DNA Kits (Catalog Numbers NA2000, NA2010, and NA202) are recommended.

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 at 10^6 particles/ml and a maximum titer at 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 be sure to remove any alcohol containing wash buffer from the preparation to avoid coelution 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:

A. Heat-inactivation of the sample material
The templates for the PCR analysis are prepared by direct heating of the cell culture supernatant or the biological sample material:

1. Transfer 100 µl of supernatant 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 95 °C for 10 minutes.
3. Briefly centrifuge (5 seconds at 1,000 × *g*) the sample supernatant to pellet cellular debris before adding to the PCR mixture. The templates are stable at 2–8 °C for at least 1 week.

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

B. Enrichment of mycoplasma by centrifugation

1. Transfer 1 ml of supernatant from the test culture to a sterile tube. The lid should be tightly sealed to prevent opening during heating.
2. Centrifuge the supernatant (15 minutes at 10,000 × *g*) to sediment mycoplasma particles. Alternatively, centrifuge the supernatant 6 minutes at 13,000 × *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 95 °C for 10 minutes.

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

The PCR Master Mix

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

	1 reaction	25 reactions
PCR Grade Water	7.6 µl	190 µl
Primer/probe/nucleotide mix	14 µl	350 µl
Internal Control DNA	1.0 µl	25 µl
Polymerase (2.5 units/µl)	0.6 µl	15.0 µl
+ template DNA, Negative control or Positive control	2.0 µl	

For other polymerase concentrations the amount of enzyme and the amount of water added to the mix need to be adjusted. Keep master mix on ice.

Pipette 23 µl of master mix into a 1.5 ml PCR reaction tube and mix gently.

The total duration from master mix preparation to PCR cycling must not exceed 60 minutes to avoid a decrease in the fluorescent signal.

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

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

Program Step 2: Amplification

Cycles	45
Denaturing	96 °C for 20 seconds
Annealing & Reading	55 °C for 30 seconds
Extension	72 °C for 30 seconds

For all vials set detectors to FAM for Mycoplasma Target Probe and to ROX for the Internal Control Probe. No quencher must be specified. Inactivate available ROX Reference functions. Fluorescence should be measured during extension.

Results

A successfully performed PCR without inhibition is indicated by an increasing fluorescence signal in the internal control channel (ROX), provided the Internal Control was added to the master mix. The Internal Control can be detected with an orange filter (585–610 nm for ROX). The presence of mycoplasmal 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.

Table 1.
Interpretation of PCR Results

Mycoplasma PCR	Internal Control	Interpretation
Positive	Irrelevant	Mycoplasma positive
Negative	Negative	PCR inhibition
Negative	Positive	Mycoplasma negative

Troubleshooting

1. No amplification of the control DNA may be due to the following:
   - programming error
   - insufficient activity of DNA *Taq* polymerase
   - reaction buffer is not suitable for polymerase used (other than JumpStart *Taq* DNA Polymerase)
   - pipetting error
2. Before rerunning of a negative and a positive control, check thermocycler program and pipetting scheme. DNA polymerase concentration can be raised up to 2.5 units/reaction.
3. If the PCR of a sample is inhibited, PCR inhibitors can easily be removed from the sample by performing a DNA extraction with the GenElute Blood Genomic DNA Kit.

Table 2.
Species specificity includes the following:

A. laidlawii	*M. agalactiae*
M. agassizii	*M. alkalescens*
M. anseris	*M. arginini*
M. arthritidis	*M. bovigenitalium*
M. bovirhinis	*M. bovis*
M. buccale	*M. buteonis*
M. californicum	*M. canadense*
M. capricolum	*M. caviae*
M. citelli	*M. cloacale*
M. collies	*M. columbinasale*
M. columbinum	*M. columborale*
M. cricetuli	*M. cynos*
M. edwardii	*M. equirhinis*
M. falconis	*M. faucium*
M. felifaucium	*M. fermentans*
M. gallinaceum	*M. gallinarum*
M. gallopavonis	*M. gateae*
M. glycophilum	*M. gypis*
M. hominis	*M. hyopharyngis*
M. hyorhinis	*M. hyosynoviae*
M. iguanae	*M. indiense*
M. iners	*M. lagogenitalium*
M. lipofaciens	*M. maculosum*
M. melegridis	*M. moatsii*
M. opalescens	*M. orale*
M. pneumoniae	*M. pulmonis*
M. salivarium	*M. simbae*
M. sp. ovine/caprine	*M. spermatophilum*
M. sphenisci	*M. spumans*
M. sualvi	*M. subdolum*
M. synoviae	*M. testudineum*
M. timone	*M. turnidae*
M. verecundum	*M. zalophi*

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