Instructions for Use

Microsart® RESEARCH Mycoplasma

Mycoplasma Detection Kit for qPCR

Prod. No. SMB95-1005 | SMB95-1006
Reagents for 25 |100 reactions
For use in research

Manufactured by:

Minerva Biolabs GmbH
Koepenicker Strasse 325
12555 Berlin
Germany

Symbols

LOT	Lot No.
REF	Order No.
	Expiry date
	Store at
	Contains reagents for 25 or 100 tests
	Manufacturer

Contents

1. Intended Use

Microsart® RESEARCH Mycoplasma is used for direct detection of Mollicutes (*Mycoplasma*, *Acholeplasma*, *Spiroplasma*) contamination in cell cultures and cell media components in research and development.

2. Explanation of the Test

Microsart® RESEARCH Mycoplasma utilizes real-time PCR (qPCR) as the method of choice. The assay can be performed with any type of real-time PCR cycler able to detect the fluorescence dyes FAM™ and ROX™. The protocol provided is preferred for the fast and reliable screening of cell culture supernatants most applicable in research and development. The detection procedure can be performed within 3 hours. In contrast to the detection by luminescence-linked enzymology, fluorescent staining methods or culture, samples do not need to contain vital mycoplasma.

3. Test Principle

Mycoplasma are specifically detected by amplifying a highly conserved rRNA operon, or more precisely, a 16S rRNA coding region in the mycoplasma genome. The mycoplasma-specific amplification is detected at 520 nm (FAM™ channel). The kit includes primers and FAM™ labeled probes which allow the specific detection of all mollicute species so far described as contaminants of cell cultures and media components. Eukaryotic DNA is not amplified by this primer/probe system.

False negative results due to PCR inhibitors or improper DNA extraction are detected by the internal amplification control which is part of the PCR master mix. The amplification of the internal amplification control is detected at 610 nm (ROX™ channel).

The kit contains dUTP instead of dTTP, so the option to degrade amplicons from previous analysis by use of uracil-DNA glycosylase (UNG) is available. Thus, the occurrence of false-positive results can be minimized. UNG is not included in the kit.

4. Reagents

Each kit contains reagents for 25 or 100 reactions. The expiry date of the unopened package is marked on the package label. The kit components are stored at +2 to +8 °C until use and must be stored at < -18 ° C after opening and rehydration. Protect the Prime Mix from light. The lot specific Certificate of Analysis can be downloaded from the manufacturer`s website (www.minerva-biolabs.com).

	Quantity		
Kit Component Label Information	25 Reactions Order No. SMB95-1005	100 Reactions Order No. SMB95-1006	Cap Color
Prime Mix	1 × lyophilized	4 × lyophilized	red
Rehydration Buffer	1 × 1.0 ml	3 × 1.0 ml	blue
Positive Control DNA	1 × lyophilized	1 × lyophilized	green
PCR grade water	1 × 1.0 ml	1 × 1.0 ml	white

5. Needed but not Included

Microsart® RESEARCH Mycoplasma contains the reagents for the specific detection of mollicutes. General industrial supplies and reagents, usually available in PCR laboratories, are not included:

- qPCR device with filter sets for the detection of the fluorescence dyes FAM™ and ROX™ and suitable for 25 µl PCR reaction volumes
- Vortexer
- PCR reaction tubes for the specific qPCR device
- Microcentrifuge for 1.5 ml PCR reaction tubes
- Pipettes with corresponding filter tips to dispense the reaction mix (10, 100 and 1000 µl)
- Optional:
  Microsart® AMP Extraction (Cat. No. SMB95-2003)
  Uracil-N-glycosylase (see page 28)

6. Specimen

Cell cultures should be tested preferably at 90 - 100 % confluence. PCR inhibiting substances may accumulate in the medium of older cultures. Penicillin and streptomycin in culture media do not inhibit mycoplasma or affect test sensitivity. Cell culture and cell culture supernatants are highly recommended to test without the need for sample preparation. Usually, with average titers of 10^6 particles/ml and a maximum titer of 10^8 particles/ml sufficient mycoplasma DNA will be present in the supernatant to guarantee a sensitive PCR even for cell wall associated species. However, please note that it is highly recommended to use preferably native cell culture samples with cells to detect intracellular mycoplasma as well.

Standard protocol:

Use 2 µl of the native cell culture or cell culture supernatant directly for PCR analysis (see 8.2).

Storage of samples for later qPCR analysis:

Samples directly retrieved from cell culture contain DNases which can degrade mycoplasma DNA even at lower temperatures. If PCR analysis cannot be performed immediately after sampling, it is advised to stabilize the sample material by freezing or heat inactivation:

1. Transfer 500 µl of cell culture or cell culture supernatant with up to 10^6 cells to a sterile reaction tube. The lid should be sealed tightly to prevent opening during heating.
2. Boil or incubate the sample at 95 °C for 10 minutes.
3. Centrifuge the sample briefly (5 seconds) at approx. 13,000 × g to pellet cellular debris.
4. The supernatant can now be used for PCR analysis.

Due to matrix effects on the sensitivity of the test, it is not recommended to use the heat-inactivated sample directly. To avoid matrix effects, use the heat-inactivated samples only after centrifugation. A DNA extraction step prior to PCR, for example using Microsart® AMP Extraction (Cat. No. SMB95-2003), is mandatory for cell pellets, cryo conservation stocks, samples from long term cultures, fetal calf serum (FCS), vaccines and paraffin-embedded samples.

If you work with native cell cultures, please note that the sample for DNA extraction can contain up to 10^6 cells/ 200 µl. Be 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.

DNA extracts and heat-inactivated samples can be stored at +2 to +8 °C for 6 days. Longer storage times require a temperature of < -18 °C. Repeated freezing and thawing should be avoided.

7. Precautions

For *in vitro* use in research. This kit should be used by trained persons only. All samples should be considered potentially infectious and handled according to local or national regulations. This kit does not contain hazardous substances and may be disposed of according to local regulations.

8. Test Procedure

The test should be carried out with negative and positive controls and samples in duplicates. For quantification, a dilution series of an appropriate standard should be prepared. All reagents and samples must be equilibrated to +2 to +8 °C prior use.

8.1 Rehydration of the reagents

After reconstitution, the reagents should be stored at < -18 °C. Repeated freezing and thawing should be avoided and the reconstituted Positive Control should be stored in aliquots.

1.	Mycoplasma Mix Positive Control DNA	red cap green cap	Spin all lyophilized components for 5 sec at maximum speed of the microcentrifuge.
2.	Mycoplasma Mix	red cap	Add 600 µl Rehydration Buffer (blue cap).
3.	Positive Control DNA	green cap	Add 300 µl PCR grade Water (white cap).
4.	Mycoplasma Mix Positive Control DNA	red cap green cap	Incubate 5 min at room temperature. Vortex briefly and spin for 5 sec.

8.2 Loading the test tubes

This process should not take more than 45 minutes to avoid a reduction in the fluorescent signal. The pipetting sequence should be followed and the tubes should be closed after each sample load.

1.	Add 23 µl to each PCR tube.
2.	Negative controls: add 2 µl PCR grade water (white cap) or elution buffer from DNA extraction kit (see chapter „Specimen“)
3.	Sample reaction: add 2 µl of sample.
4.	Positive control: add 2 µl Positive Control DNA (green cap).
5.	Close tightly and spin all PCR tubes briefly.

8.3 Starting the reaction

1. Load qPCR cycler and check each PCR tube and the cycler lid for tight fit.
2. Program the qPCR cycler or check stored temperature profiles.
   See Appendix for temperature profiles of selected qPCR cyclers. Programs for additional cyclers might be available on request.
3. Start the program and data reading.

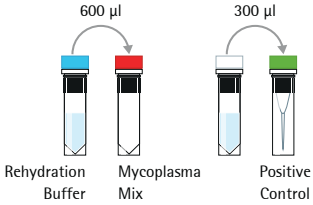
8.4 Analysis

1. Safe the data at the end of the run.
2. Read the channels for the fluorescence dyes FAM™ and ROX™ and show the 2nd deviation of the data.
3. Set the threshold following the standard routine of the cycler or for selected cyclers as described in the Appendix. Check the fluorescence data for each sample for typical amplification curves (logarithmic increase of fluorescence) and correct threshold setting manually, if needed.
4. Read the calculation of the Ct-values for negative controls, positive controls and samples.

9. Short Instructions

1. Rehydration of Reagents

Mycoplasma Mix
and Positive Control

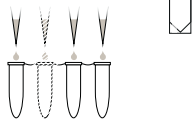


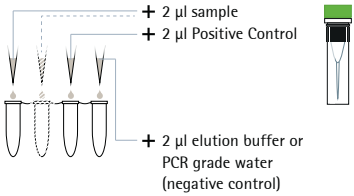
for 5 min RT
briefly
for 5 sec

2. Preparation of PCR Reactions

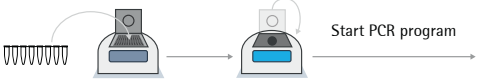
loading the test tubes

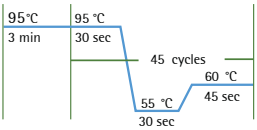
+23 µl Mycoplasma Mix





3. Starting the PCR Reaction





Rehydration Buffer
Mycoplasma Mix
PCR grade water
Positive Control DNA

incubate
vortex
centrifuge
add

storage 2-8 °C
after rehydration < -18 °C

This procedure overview is not a substitute for the detailed manual.

ST_SI_Microsart®-RESEARCH-Mycoplasma_01_EN

10. Notes on the Test Procedure

1. This leaflet must be widely understood for a successful use of Microsart® RESEARCH Mycoplasma kit. The reagents supplied should not be mixed with reagents from different lots but used as an integral unit. The reagents of the kit should not be used beyond their shelf life.

2. Any deviation from the test method can affect the results.

3. Inhibition may be caused by the sample matrix, but also by sample elution buffer of DNA extraction kits which are not recommended or validated. If DNA extraction was carried out, the negative controls should always be set up with the elution buffer used for the DNA extraction.

4. For each test setup, at least one negative control should be included. Positive controls facilitate the evaluation of the test. Typical Ct-values for the internal control and positive control are shown on the Certificate of Analysis and can be used as a guideline for quality control.

5. The use of control samples is advised to secure the day-to-day validity of results. The controls should be processed in the same manner as the samples. It is recommended to run laboratory specific control samples with a high, medial and low level (e.g. 3 × LOD_{95}), or established commercial controls, for example Sartorius Microsart® Calibration Reagents (see page 28). We recommend the participation in external quality control programs, as offered biannually by Minerva Biolabs.

11. Interpretation of Results

The presence of mycoplasma DNA in the sample is indicated by an increasing fluorescence signal in the FAM™ channel during PCR. A successfully performed PCR without inhibition is indicated by an increasing fluorescence signal in the internal control (ROX™) channel. Mycoplasma DNA and Internal Control DNA are competitors in PCR. Because of the very low concentration of Internal Control DNA in the PCR mix, the signal strength in this channel is reduced with increasing mycoplasma DNA loads in the sample.

Detection of Mollicutes FAM™ channel	**Internal control ROX™ channel**	**Interpretation**
positive (Ct < 40)	irrelevant	Mollicutes positive
negative (no Ct)	negative (no Ct)	PCR inhibition
negative (no Ct)	positive (Ct < 40)	Mollicutes negative
borderline (Ct > 40)	positive (Ct < 40)	result not valid, repeat process including DNA extraction
borderline (Ct > 40)	negative (no Ct)	PCR inhibition

All samples showing a Ct value of > 40 need to be evaluated manually. Check the amplification curves for a significant increase of the fluorescence signal in comparison to the background noise of the negative control. In case of amplification, the curve should form a typical logarithmic "amplification" curve. Adjust the threshold cycle manually. However, it is advised to repeat the testing of samples showing a borderline signal, as such a signal is not necessarily indicating the amplification of mycoplasma DNA but can be caused by sample matrix effects caused by incorrect sample preparations or setup errors.

12. Appendix

The assay of this kit can be performed with any type of real-time PCR cycler able to detect the fluorescence dyes FAM™ and ROX™.

The Microsart® RESEARCH Mycoplasma kit was successfully tested for the following devices:

LightCycler® 1.0 and 2.0
RotorGene® 6000
ABI Prism® 7500
Mx3005P®
CFX96 Touch™ / CFX96 Touch™ Deep Well
AriaMx

LightCycler® 1.0 and 2.0

Program 1: Pre-incubation	
Cycles	1
Analysis Mode	None
Temperature Targets	**Segment 1**
Target Temperature [°C]	95
Incubation time [s]	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 LC 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]	10	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 [s]	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 *Channel 3* (610 nm)
- Click on *Quantification* to generate the amplification plots and the specific Ct-values
- The threshold will be generated automatically.

RotorGene® 6000 (5-plex)

1. Check the correct settings for the filter combination:

Target	Mollicutes	Internal Control
filter	green	orange
wavelength	470—510 nm	585-610 nm

2. Program the Cycler

Program 1: Pre-incubation	
Setting	Hold
Hold Temperature	95 °C
Hold Time	3 min 0 sec

Program Step 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 orange)
Gain setting	automatic (Auto-Gain)
Slope Correct	activated
Ignore First	deactivated

3. Analysis:

- Open the menu *Analysis*
– Select *Quantitation*
– Check the required filter set (green and orange) according to the following table and start data analysis by double click.
– The following windows will appear:
Quantitation Analysis - Cycling A (green / orange)
Quant. Results - Cycling A (green / orange)
Standard Curve - Cycling A (green / orange)
– 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 Ct-values can be taken from the window *Quant. Results*.
– Samples showing no Ct-value can be considered as negative.

ABI Prism® 7500

1. Check the correct settings for the filter combination:

Target	Mollicutes	Internal Control
filter	FAM	ROX
wavelength	470—510 nm	585-610 nm
quencher	none	none

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

2. Program the Cycler

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

Program Step 2: Amplification	
Cycles	45
Setting	Cycle
Denaturing	95 °C for 30 sec
Annealing	55 °C for 30 sec
Extension	60 °C for 45 sec

3. Analysis:

- Enter the following basic settings at the right task bar:
  Data: *Delta RN vs. Cycle*
  Detector: *FAM and ROX*
  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 Ct-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 Ct-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 ROX
- 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: 1 cycle	3 min	95 °C	
Segment 2: 45 cycles	30 sec	95 °C	
	30 sec	55 °C	
	45 sec	60 °C	data collection end

Analysis mode: non adaptive baseline (baseline correction)
- at menu *Run Status* select *Run* and start the cycler by pushing *Start*

Analysis of raw data:
- In the window *Analysis* tab on *Analysis Selection / Setup* to analyse 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 Ct-values at *Text Report*

Bio-Rad CFX96 Touch™ / CFX96 Touch™ deep well

Run Setup Protocol Tab:

- Click *File* --> *New* --> *Protocol* to open the Protocol Editor to create a new protocol
- Select any step in either the graphical or text display
- Click the temperature or well time to directly edit the value

Segment 1:	1 cycle	3 min	95 °C	
Segment 2:		30 sec	95 °C	
Segment 3:		30 sec	55 °C	
Segment 4:		45 sec	60 °C	data collection
		GO TO Step 2, 44 more cycles		

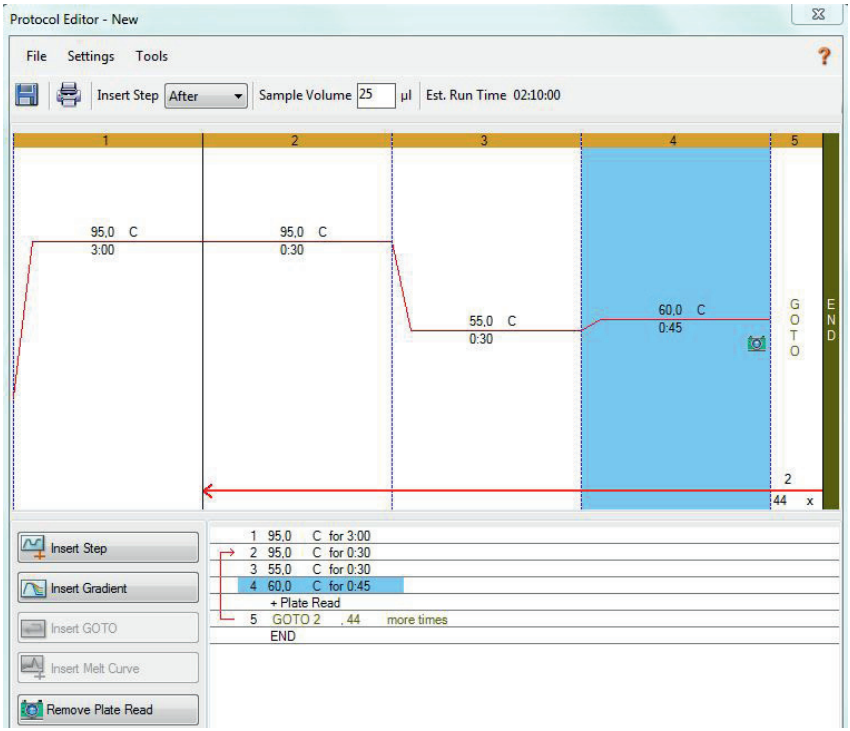
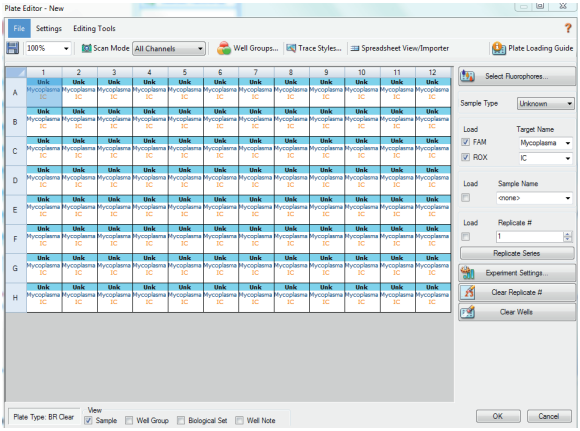


Plate Setup:

- Click *File* --> *New* --> *Plate* to open the Plate Editor to create a new plate
- Specify the type of sample at *Sample Type*
- Name your samples at *Sample Name*
- Use the *Scan Mode* dropdown menu in the Plate Editor Toolbar to designate the data acquisition mode to be used during the run. Select *All Channels* mode
- Click *Select Fluorophores* to indicate the fluorophores that will be used in the run. Choose FAM for the detection of mycoplasma amplification and ROX for monitoring the amplification of the internal control. Within the plate diagram, select the wells to load
- Choose the fluorophore data you want to display by clicking the fluorophore checkboxes located under amplification chart. Select FAM to display data of mycoplasma detection and ROX to display internal control amplification data.



Data Analysis:

- Select *Settings* in the menu and select *Baseline Subtracted Curve Fit* as baseline setting and *Single Threshold mode* as Cq determination
- View amplification curves of FAM channel by selecting the FAM checkbox under the amplification plot
- By right-clicking inside the amplification plot choose *Baseline Threshold* and set baseline cycles manually on basis of your positive control. Set *Baseline Begin* when fluorescence signal levelled off at a constant level. Set *Baseline End* before fluorescence signal of positive control increases
- Drag the threshold line manually to the initial linear section of the positive control
- Note specific Ct values

Agilent AriaMx

- Click on *New Experiment* and *Quantitative PCR - Fluorescence Probe*
- Go to the *Plate Setup* menu and check all positions which apply
- Choose the fluorophore data you want to display by clicking the fluorophore checkboxes located under *Well Types* drop down menu. Select FAM (blue) to display data of mycoplasma detection and ROX (orange) to display internal control amplification data.
- Corresponding to the basic settings the *Reference Dye* function should be deactivated
- Use the *Well Types* drop down menu to specify the type of sample
- Name the samples
- Edit the temperature profile by changing to *Thermal profile* tab

Segment 1:	1 cycle	3 min	95 °C	
Segment 2:	45 Cycles	30 sec	95 °C	
		30 sec	55 °C	
		45 sec	60 °C	data collection end

- Start the Run by clicking *Run Experiment* in the Thermal Profile tab in the top right-hand corner

Data Analysis:

- In the area *Analysis* click on *Analysis Criteria* and mark the wells you want to analyze
- By clicking the checkbox at the top left-hand of the plate screen you can select all wells
- By changing to *Graphical Display* tab you can see the amplification plot
- Choose *ΔR* (baseline corrective raw fluorescence) under *Fluorescence Term* and turn the *Smoothing* option on
- The Threshold will be generated automatically
- The automatic threshold line should be adapted to the initial linear section of the positive control. If this is not the case change to *Log* view by clicking the triangle under *Smoothing* options to see advanced options for the amplification plot. Choose the *Log* option for the *graph type* and adapt the threshold line manually to the middle of the linear section of the positive control
- Read specific Ct-values at *Result table*

Appendix

Limited Product Warranty
This warranty limits our liability for replacement of this product. No warranties of any kind, express or implied, including, without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided. Minerva Biolabs shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

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LightCycler is a registrated trademark of a member of the Roche Group. ABI Prism is a registrated trademark of Applera Corporation or its subsidiaries in the US and certain other countries. RotorGene is a registered trademark of Corbett Life Science. FAM™ and ROX™ are trademarks of Applera Corporation or it's subsidiaries in the US and certain other countries. Mx3005P is a trademark of Agilent Technologies. CFX96 Touch is a trademark of Bio Rad Laboratories, Inc. Microsart is a registered trademark of Sartorius Stedim Biotech. Myocoplasma Off and PCR Clean are trademarks of Minerva Biolabs GmbH, Germany.

Last technical revision: 06-2017

13. Related products

Detection Kits for qPCR

SMB95-1001/1002	Microsart® AMP Mycoplasma	25/100 tests
SMB95-1003/1004	Microsart® ATMP Mycoplasma	25/100 tests
SMB95-1005/1006	Microsart® RESEARCH Mycoplasma	25/100 tests

Microsart® Calibration Reagent, 1x10^8 genomes

SMB95-2021	Mycoplasma arginini
SMB95-2022	Mycoplasma orale
SMB95-2023	Mycoplasma gallisepticum
SMB95-2024	Mycoplasma pneumoniae
SMB95-2025	Mycoplasma synoviae
SMB95-2026	Mycoplasma fermentans
SMB95-2027	Mycoplasma hyorhinis
SMB95-2028	Acholeplasma laidlawii
SMB95-2029	Spiroplasma citri

Microsart® Validation Standard, 3 vials each

SMB95-2011	Mycoplasma arginini
SMB95-2012	Mycoplasma orale
SMB95-2013	Mycoplasma gallisepticum
SMB95-2014	Mycoplasma pneumoniae
SMB95-2015	Mycoplasma synoviae
SMB95-2016	Mycoplasma fermentans
SMB95-2017	Mycoplasma hyorhinis
SMB95-2018	Acholeplasma laidlawii
SMB95-2019	Spiroplasma citri

DNA Extraction Kit

SMB95-2003	Microsart® AMP Extraction	50 extractions

PCR Clean™ *

15-2025	DNA Decontamination Reagent, spray bottle	250 ml
15-2200	DNA Decontamination Reagent, refill bottles	4x 500 ml

Mycoplasma Off® *

15-1000	Surface Disinfectant Spray, spray bottle	1000 ml
15-5000	Surface Disinfectant Spray, refill bottles	5x 1000 ml

Mycoplasma Off® Wipes*

15-1001	Surface Disinfectant Wipes	120 wipes
15-5001	Surface Disinfectant Wipes, refill sachets	5 x 120 wipes

UNG Carry over prevention*

54-1001	Uracil-DNA Clycosylase (UNG), heat-labile	100 u, 1 u/µl

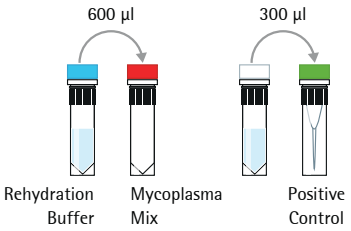
*** Distributed by Minerva Biolabs**

Procedure-Overview

Microsart® RESEARCH Mycoplasma

1. Rehydration of Reagents

Mycoplasma Mix
and Positive Control

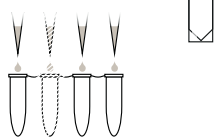


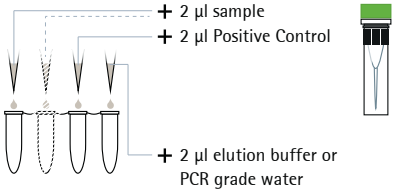
for 5 min RT
briefly
for 5 sec

2. Preparation of PCR Reactions

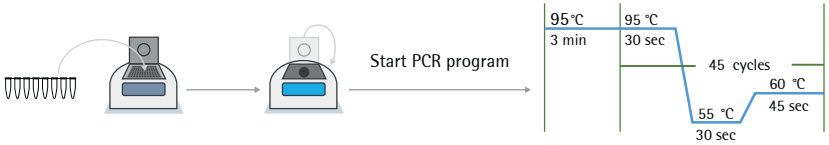
loading the test tubes

+ 23 µl Mycoplasma Mix





3. Starting the PCR Reaction



Rehydration Buffer
Mycoplasma Mix
PCR grade water
Positive Control DNA

incubate
vortex
centrifuge
+ add

storage 2-8 °C
after rehydration < -18 °C

This procedure overview is not a substitute for the detailed manual.

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