Instructions for Use

Microsart® AMP Mycoplasma

Mycoplasma Detection Kit for qPCR

Prod. No. SMB95-1001 | SMB95-1002
Reagents for 25 |100 reactions
For use in research and quality control

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® AMP Mycoplasma is used for the direct detection of Mollicutes (*Mycoplasma*, *Acholeplasma*, *Spiroplasma*) in cell cultures, cell culture media components and cell culture derived biologicals according to European Pharmacopoeia (EP) 2.6.7 "Mycoplasmas".

2. Explanation of the Test

Microsart® AMP Mycoplasma utilizes real-time PCR (qPCR) as the method of choice for sensitive and robust detection of mycoplasma contaminations. The assay can be performed with any type of real-time PCR cycler able to detect the fluorescence dyes FAM™ and ROX™. 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.

The kit was validated according to EP 2.6.7 "Mycoplasmas" in combination with EP 2.6.21 "Nucleic Acid Amplification Techniques" with respect to detection limit for all listed mycoplasma species, specificity and robustness for cell cultures and autologous cell transplants (e.g. chondrocytes). The kit complies fully with the requirements of EP 2.6.7. The validation report is available on request. However, please note that these validation data are provided for information purposes only. EP 2.6.7 clearly states "Where commercial kits are used ..., documented validation points already covered by the kit manufacturer can replace validation by the user. Nevertheless, the performance of the kit with respect to its intended use has to be demonstrated by the user (e.g. detection limit, robustness, cross-detection of other classes of bacteria)". Please feel free to contact us if you need further assistance.

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 mentioned in EP 2.6.7 and many more. The polymerase is part of the Mycoplasma Mix.

False negative results due to PCR inhibitors or improper DNA extraction are detected by the internal amplification control. The Internal Control DNA can be added directly to the PCR master mix to act as a PCR control or used to monitor the extraction process. 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 specified 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 Mycoplasma 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. SMB95-1001	100 Reactions Order No. SMB95-1002	Cap Color
Mycoplasma Mix	1 × lyophilized	4 × lyophilized	red
Rehydration Buffer	1 × 1.5 ml	4 × 1.5 ml	blue
Positive Control DNA	1 × lyophilized	4 × lyophilized	green
Internal Control DNA	1 × lyophilized	4 × lyophilized	yellow
PCR grade Water	1 × 1.0 ml	4 × 1.0 ml	white

5. Needed but not Included

Microsart® AMP Mycoplasma contains all the reagents, including negative and positive controls, and polymerase as a component of the Mycoplasma Mix to perform the test. 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 100 µl PCR reaction volumes
- Vortexer
- PCR reaction tubes for the specific qPCR device
- 1.5 ml reaction tubes, DNA- and RNA-free
- Microcentrifuge for 1.5 ml PCR reaction tubes
- Pipettes with corresponding filter tips to prepare and dispense the reaction mix (10, 100 and 1000 µl)
- Optional:
  Microsart® AMP Extraction (Cat. No. SMB95-2003)
  Vivaspin® 6 or 20 (Cat. No. VS0641 / VS2041) and compatible centrifuges
  Microsart® AMP Coating Buffer (Cat. No. SMB95-2002)
  Microsart® Validation Standard available for all EP-listed mycoplasma species (see page 25)
  Microsart® Calibration Reagent available for all EP-listed mycoplasma species (see page 25)
  Uracil-N-glycosylase (see page 25)

6. Specimen for Compliant Testing

6.1 Sample collection and concentration

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 200 µl of the native cell culture or cell culture supernatant directly for DNA extraction (6.3).

Concentration step for sample volumes > 200 µl:
For samples from 200 µl to 18 ml an enrichment step is recommended to take advantage of the large sample volume and to increase the sensitivity of the test. Please note that this protocol is suitable for native samples only. For the following test procedures the Microsart® AMP Extraction kit (Cat. No. SMB95-2003) is recommended. This kit was especially designed to complement the Microsart® AMP Mycoplasma qPCR. Please note that the concentrated sample used for DNA extraction can contain up to 10^6 cells / 200 µl.

Test procedure for sample volumes > 200 µl to 5 ml

1.	Add 1 ml Microsart® Coating Buffer to the sample and vortex the mixture briefly. Transfer the mixture into a Vivaspin® 6 unit.
2.	Centrifuge for up to 30 min depending on the maximum speed of the available centrifuge and the viscosity of the sample matrix (see Vivaspin® operation instructions for details). The dead stop volume of the Vivaspin® 6 is 30 µl. The sample is concentrated to a volume < 200 µl.
3.	Estimate the volume of the retentate during transfer into a fresh reaction tube.
4.	Rinse the Vivaspin® 6 unit with the lysis buffer of the DNA extraction kit (Buffer A1 in case of Microsart® AMP Extraction). The wash volume depends on the volume of the retentate. In case of Microsart® AMP Extraction: wash volume = 400 µl – volume retentate. The wash is added to the retentate.

5. The complete sample can be used for DNA extraction. The sample (400 µl) contains the starting material (cell culture) and the lysis buffer. Proceed with adding buffer A2 and follow further instructions according to Microsart® AMP Extraction manual.

Test procedure for sample volumes > 5 to 18 ml

1. Add 2 ml Microsart® AMP Coating Buffer to the sample and vortex the mixture briefly. Transfer the mixture into a Vivaspin® 20 unit.
2. Centrifuge for up to 40 min depending on the maximum speed of the available centrifuge and the viscosity of the sample matrix (see Vivaspin® operation instructions for details). The dead volume of the Vivaspin® 20 is 50 µl. The sample is concentrated to a volume < 200 µl.
3. Estimate the volume of the retentate during transfer into a fresh reaction tube.
4. Rinse the Vivaspin® 20 unit with the lysis buffer of the DNA extraction kit (Buffer A1 in case of Microsart® AMP Extraction). The wash volume depends on the volume of the retentate. In case of Microsart® AMP Extraction:
wash volume = 400 µl – volume retentate. The wash is added to the retentate.
5. The complete sample can be used for DNA extraction. The sample (400 µl) contains the starting material (cell culture) and the lysis buffer. Proceed with adding buffer A2 and follow further instructions according to Microsart® AMP Extraction manual.

6.2 Sample storage for later DNA extraction

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 DNA extraction (max. 200 µl).

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.

6.3 DNA extraction

Studies showed the strict requirement of DNA extraction for any kind of sample to achieve highest sensitive testing. For most test materials a DNA extraction method is available providing templates suitable for PCR. However, the DNA extraction method should be applicable for mycoplasma genomes. The resulting template must be validated in combination with the kit. For DNA extraction we recommend the Microsart® AMP Extraction kit (Cat. No. SMB95-2003), which was validated intensively as integral part of the testing procedure. The protocol for DNA extraction is described in detail in the manual of the DNA extraction kit.

Optional: According to EP 2.6.7, a sensitivity of 10 CFU/ml must be reached. The sample material can be spiked with mycoplasma particles by using special reference materials (10CFU™ Sensitivity Standards, see page 25) and processed in parallel.

Recommended: The Internal Control DNA of Microsart® AMP Mycoplasma can be used to monitor the extraction process. Add 5 µl per 60 µl DNA extract directly to the sample, vortex briefly and process the DNA extraction as described. Please note, that the sample volume to be spiked is irrelevant for the required volume of Internal Control DNA. No additional Internal Control DNA is required in the reaction mix.

Extracts can be stored at +2 to +8 °C for 6 days. Longer storage periods require a temperature of < -18 °C. Repeated freezing and thawing should be avoided.

7. Precautions

For *in vitro* use in research and quality control. 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 (> 4 cycles) should be avoided or, if not avoidable, reagents should be stored in aliquots.

1.	Mycoplasma Mix Internal Control DNA Positive Control DNA	red cap yellow cap green cap	Spin all lyophilized components for 5 sec at maximum speed of the centrifuge
2.	Mycoplasma Mix	red cap	Add 1275 µl Rehydration Buffer (blue cap)
3.	Internal Control DNA	yellow cap	Add 300 µl PCR grade Water (white cap)
4.	Positive Control DNA	green cap	Add 300 µl PCR grade Water (white cap)
5.	Mycoplasma Mix Internal Control DNA Positive Control DNA	red cap yellow cap green cap	Incubate 5 min at room temperature
6.	Mycoplasma Mix Internal Control DNA Positive Control DNA	red cap yellow cap green cap	Vortex briefly and spin for 5 sec

8.2 Preparation of the Reaction Mix

Preparation of the reaction mix and sample loading should not take longer than 45 min to avoid a reduction in the fluorescence signal. The pipetting sequence should be followed strictly and the tubes should be closed after each sample load.

If the Internal Control DNA was not added to the sample to monitor the DNA extraction process, follow this protocol:

1. Prepare the required amount of reaction mix at room temperature in a 1.5 ml reaction tube for all control and test reactions.

	Cap color	For 1 reaction	For 25 reactions
Mycoplasma Mix	red	49.0 µl	1225.0 µl
Internal Control DNA	yellow	1.0 µl	25.0 µl

2. Homogenize the reaction mix by tapping carefully against the tube.

3. Add 50 µl to each PCR tube.

If the Internal Control DNA was added to the sample prior to DNA extraction add 50 µl of the Mycoplasma Mix (red cap) directly to each PCR tube.

8.3 Loading the Test Tubes

1. Negative Controls: add 50 µl elution buffer from DNA extraction kit (see chapter "Specimen") or 50 µl PCR grade Water (white cap)
2. Sample: add 50 µl of sample
3. Positive Control: add 50 µl Positive Control DNA (green cap)
4. Close tightly and spin all PCR tubes briefly.

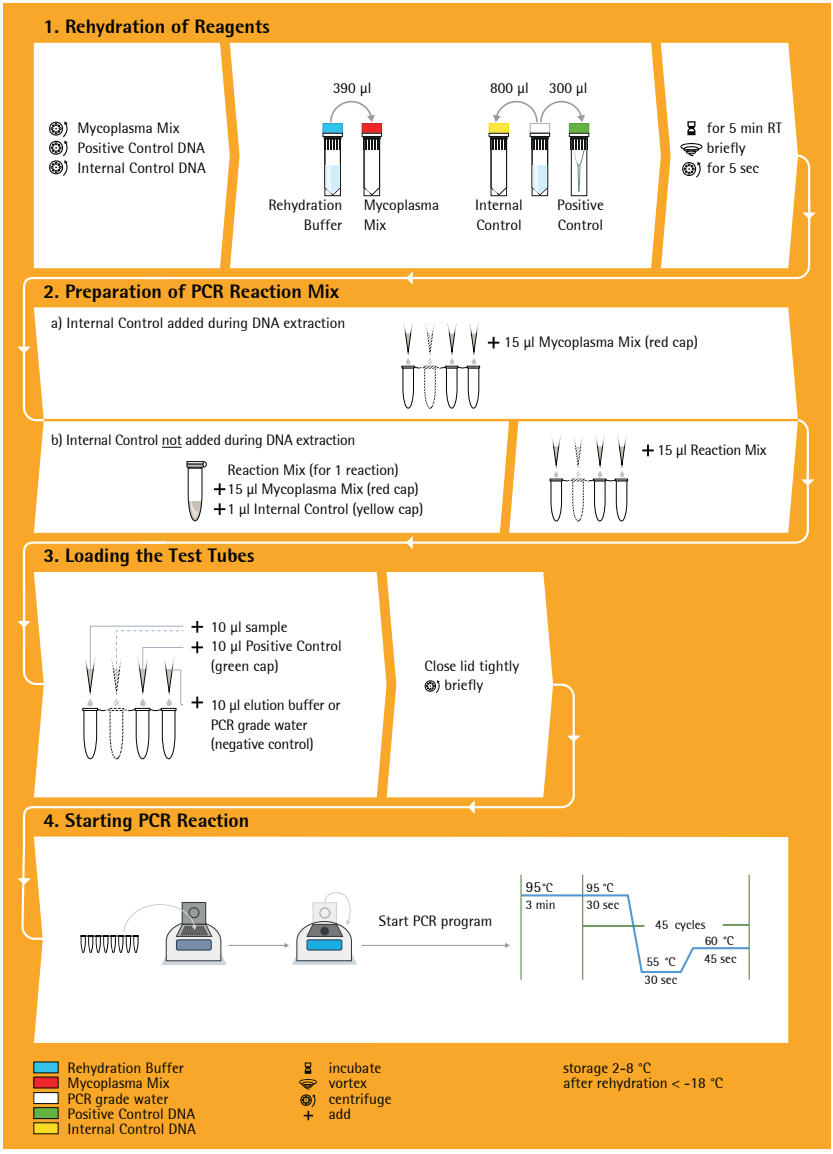
8.4 Starting the Reaction

1. Load the qPCR cycler and check each PCR tube and the cycler lid for tight fit.
2. Program qPCR cycler or apply an appropriate stored temperature profile. 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.

8.5 Analysis

1. Save the data at the end of the run.
2. Analyse 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. Analyse the calculation of the Ct-values for the negative controls, positive controls and samples.

9. Short Instructions



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

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10. Notes on the Test Procedure

1. This leaflet must be widely understood for a successful use of the Microsart® AMP 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 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 recom mended 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® Validation Standards or Microsart® Calibration Reagents, available for all mycoplasma species listed in EP 2.6.7 (see page 25). We recommend the participation in external quality control programs, as offered biannually by Minerva Biolabs.

11. Interpretation of Results

The presence of mollicutes 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 channel. Mollicute 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 mollicute DNA load 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 following qPCR cyclers were used for the validation of Microsart® AMP Mycoplasma kit:

ABI Prism® 7500
Mx3005P®

According to the device specifications of the manufacturer, the following cycler is compatible with the Microsart® AMP Mycoplasma assay:

CFX96 Touch™ deep well

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	(Pre-Melt)	3 min	95 °C	
Segment 2	45 cycles	30 sec	95 °C	
		30 sec	55 °C	
		45 sec	60 °C	data collection

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		

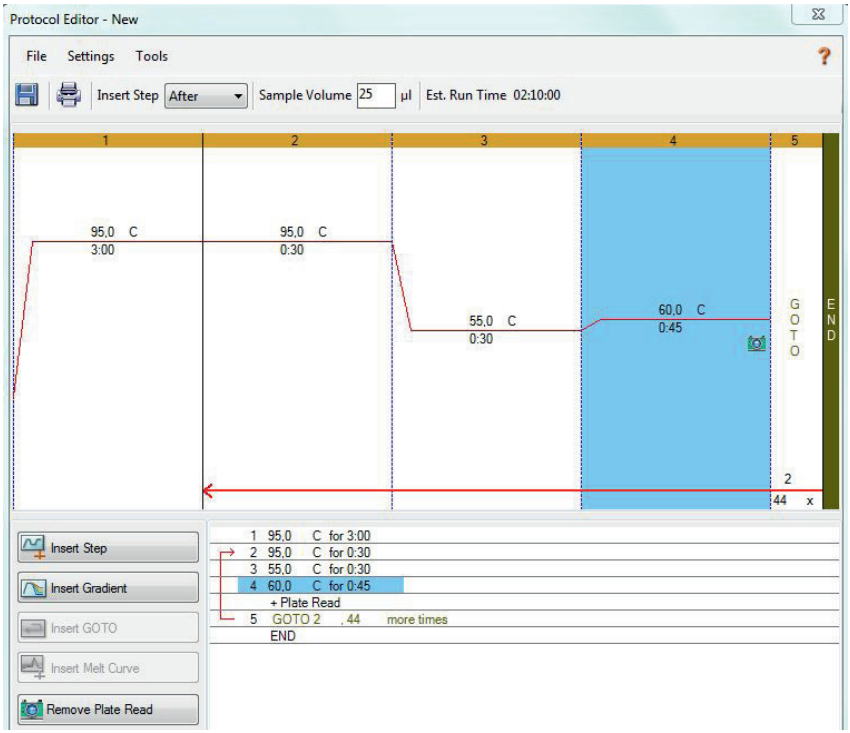
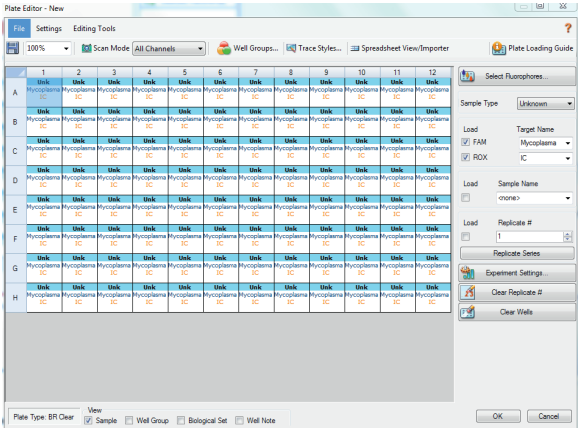


Plate Setup:

- 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

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. Sartorius Stedim Biotech GmbH and 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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Last technical revision: 2017-06

12. 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

Vivaspin® and Coating Buffer

SMB95-2002	Microsart® AMP Coating Buffer	20x 2 ml
VS0641	Vivaspin® 6 Polyethesulfone 100,000 MWCO	25 units
VS0642	Vivaspin® 6 Polyethesulfone 100,000 MWCO	100 units
VS2041	Vivaspin® 20 Polyethesulfone 100,000 MWCO	12 units
VS2042	Vivaspin® 20 Polyethesulfone 100,000 MWCO	48 units

Microsart® Calibration Reagent, 1x10^8 genomes/vial

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*

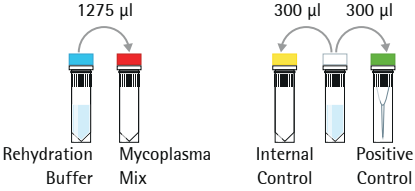
54-1001	Uracil-DNA Clycosylase (UNG), heat-labile	100 u, 1 u/µl

*** Distributed by Minerva Biolabs**

Procedure-Overview

1. Rehydration of Reagents

- Mycoplasma Mix
- Positive Control DNA
- Internal Control DNA



- for 5 min RT
- briefly
- for 5 sec

2. Preparation of PCR Reaction Mix

a) Internal Control added during DNA extraction

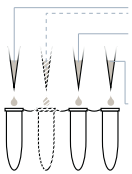
+ 50 µl Mycoplasma Mix (red cap)

b) Internal Control not added during DNA extraction

+ Reaction Mix (for 1 reaction)
+ 49 µl Mycoplasma Mix (red cap)
+ 1 µl Internal Control (yellow cap)

+ 50 µl Reaction Mix

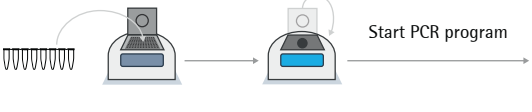
3. Loading the Test Tubes



+ 50 µl sample
+ 50 µl Positive Control (green cap)
+ 50 µl elution buffer or PCR grade water (negative control)

Close lid tightly
briefly

4. Starting PCR Reaction



Rehydration Buffer
Mycoplasma Mix
PCR grade water
Positive Control DNA
Internal Control DNA

incubate
vortex
centrifuge
add

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

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in this manual is indicated by
the date given below.
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specifications and design of the
equipment without notice.

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