

Microsart® ATMP Mycoplasma

Mycoplasma Detection Kit for qPCR
Prod. No. SMB95-1003 | SMB95-1004

Reagents for 25 | 100 reactions
For use in research and quality control

Manufactured by:



Minerva Biolabs GmbH | Schkopauer Ring 13 | 12681 Berlin | Germany

Symbols

LOT

Lot No.

REF

Order No.



Expiry date



Store at



Contains reagents for
25 or 100 reactions



Manufacturer

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1. Intended Use

Microsart® ATMP Mycoplasma is used for the direct detection of Mollicutes (Mycoplasma, Acholeplasma, Spiroplasma) in cell cultures and cell culture derived biologicals, like Advanced Therapy Medicinal Products (ATMPs), according to European Pharmacopoeia (EP) 2.6.7 “Mycoplasmas”.

2. Explanation of the Test

Microsart® ATMP 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 cyclers able to detect the fluorescent dyes FAM™ and ROX™. The detection procedure can be performed within 3 hours. In contrast to the detection by luminescence-based enzyme assays, fluorescent staining, or culture methods, samples do not need to contain living 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 specific detection of all mollicute species mentioned in EP 2.6.7 and more. The Mycoplasma Mix contains the polymerase.

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 control is detected at 610 nm (ROX™ channel).

The kit contains dUTP instead of dTTP, in order to degrade amplicons from previous analysis by using uracil-DNA glycosylase (UNG). This minimizes the occurrence of false-positive results. UNG is not included in the kit.

4. Notes on the Test Procedure

1. For in vitro use in research and quality control. This kit should be used by trained staff, only.
2. 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.
3. This leaflet must be fully understood in order to successfully use Microsart® ATMP 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.
4. Any deviation from the test method can affect the results.
5. Assay inhibition may be caused by the sample matrix, but also by sample elution buffers of incompatible or non-validated DNA extraction kits. If DNA extraction is performed, the negative controls should always be prepared with the elution buffer used for DNA extraction.
6. 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.
7. The use of control samples is advised to ensure the day-to-day validity of results. The controls should be handled 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 LOD95), 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 Related Products).
8. We recommend the participation in external quality control programs, such as those offered by Minerva Biolabs.

5. 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 Mycoplasma Mix from light. The lot specific Certificate of Analysis can be downloaded from the manufacturer's website (www.minerva-biolabs.com).

Kit Component Label Information	Quantity		Cap Color
	25 Reactions Order No. SMB95-1003	100 Reactions Order No. SMB95-1004	
Mycoplasma Mix	1 × lyophilized	4 × lyophilized	red
Rehydration Buffer	1 × 0.5 ml	4 × 0.5 ml	blue
Positive Control DNA	1 × lyophilized	1 × lyophilized	green
Internal Control DNA	1 × lyophilized	4 × lyophilized	yellow
PCR grade Water	1 × 1.5 ml	4 × 1.5 ml	white

6. Needed but not included

Microsart® ATMP 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 25 µl PCR reaction volumes
- Vortex
- 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 (Prod. No. SMB95-2003)
- Microsart® Validation Standard available for all EP- listed mycoplasma species (see Related Products)
- Microsart® Calibration Reagent available for all EP- listed mycoplasma species (see Related Products)
- 10 mM Tris buffer, pH 8.4

7. Specimen

7.1 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. The kit has been validated using a collection of several representative cell culture samples (e.g. cell supernatants, suspensions etc.) provided by our customers. The assay can be successfully performed with different types of cell culture-derived material or ATMPs. Therefore, the maximum cell number can vary significantly according to the specific characteristics of the sample (e.g. medium, cell type, and a combination of both factors) and suitability should be checked as part of each matrix specific validation.

Standard protocol:

Use 200 μ l of the native cell culture or cell culture supernatant directly for DNA extraction (see 7.3).

Concentration step for 1 ml sample volume:

When an increase in the test sensitivity is needed, we recommend processing 1 ml of sample volume. In this regard, an enrichment step is required. Please note that this protocol is suitable for native samples only. For the following test procedures the Microsart® AMP Extraction kit (Prod. No. SMB95-2003) is recommended. This kit was especially designed to complement the Microsart® ATMP Mycoplasma qPCR.

-
1. Transfer 1 ml of cell culture or cell culture supernatant into a sterile reaction tube.

 2. Centrifuge the sample at $10,000 \times g$ for 15 min to sediment mycoplasma particles. Alternatively: centrifuge the sample for 6 min at $13,000 \times g$.

 3. Discard the supernatant and suspend the pellet in 200 μ l buffer (10 mM Tris, pH 8.4).

 4. Vortex the sample and use for DNA extraction.

7.2 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 an appropriate volume (e.g. 500 µl) of cell culture or cell culture supernatant to a sterile reaction tube. The lid should be sealed tightly to prevent opening during heating.
2. Incubate the sample at 95 °C for 10 min.
3. Centrifuge the sample briefly (5 sec) 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 requires a temperature of ≤ -18 °C. Repeated freezing and thawing should be avoided.

7.3 DNA extraction

Several studies show that DNA extraction is required to achieve highest sensitivity during testing of any kind of sample. For most types of specimen, a standard DNA extraction method can be applied to obtain suitable PCR templates. However, such DNA extraction method must be applicable to the purification of mycoplasma genomic DNA. Furthermore, the obtained template must be validated in combination with the kit. Therefore, for an optimal performance, we recommend Microsart® AMP Extraction kit (Prod. No. SMB95-2003), which was extensively validated in combination with this testing procedure. The protocol for DNA purification is described in detail in the manual of the DNA extraction kit.

Optional: The EP 2.6.7 requires a test sensitivity of 10 CFU/ml. Therefore, the sample can be spiked with mycoplasma particles by using special reference materials (Microsart® Validation Standards, Mollicutes see Related Products) and processed in parallel.

Recommended: The Internal Control DNA of Microsart® ATMP Mycoplasma can be used to monitor the extraction process. Add 2 µl per 10 µl DNA extract directly to the sample, vortex briefly and proceed to the DNA extraction as described. Please note that only the final elution volume is relevant here, whereas the sample volume to be used does not affect the amount of Internal Control DNA. Once added, the Internal Control DNA does not need to be pipetted in the reaction mix any longer (see chapter 8.2). Extracts can be stored at +2 to +8 °C for 6 days. Longer storage requires a temperature of ≤ -18 °C. Repeated freezing and thawing should be avoided.

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 to 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 reconstituted controls (Internal Control DNA and Positive Control DNA) should be stored in aliquots.

Note only for the 10-reactions sample kit: please reconstitute the Mycoplasma Mix (10rxn) as indicated below (Note to step 2.). All the other steps of the test procedure do not differ for this kit format.

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
2.	Mycoplasma Mix	red cap	Add 390 μ l Rehydration Buffer (blue cap) Alternative for 10-reactions sample kit: Add 160 μ l Rehydration Buffer (blue cap)
3.	Internal Control DNA	yellow cap	Add 800 μ l PCR grade Water (white cap) For StepOne™ / StepOne Plus™: If the ROX™ signals are too low, add 160 μ l instead of 800 μ l to rehydrate the Internal Control DNA (yellow cap).
4.	Positive Control DNA	green cap	Add 300 μ l 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

The preparation of the reaction mix and sample loading should not take longer than 45 minutes to avoid a reduction in the fluorescent signal. The pipetting sequence should be followed strictly and the tubes should be closed after each sample has been loaded.

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	15.0 μ l	375.0 μ l
Internal Control DNA	yellow	1.0 μ l	25.0 μ l

2. Mix by gently tapping the tube.

3. Add 15 μ l to each PCR tube.
-

If the Internal Control DNA was added to the sample prior to DNA extraction, add 15 μ l of the Mycoplasma Mix (red cap) directly to each PCR tube.

8.3 Addition of samples and controls

1. Negative controls: add 10 µl elution buffer from DNA extraction kit (see chapter „Specimen“) or PCR grade Water (white cap).
 2. Sample reaction: add 10 µl of sample.
 3. Positive control: add 10 µl Positive Control DNA (green cap).
-

Important: If the Internal Control DNA was added to the samples during DNA extraction, add 1 µl of Internal Control DNA to each negative and positive control.

4. Close tightly and spin all PCR tubes briefly.
-

8.4 Start of the qPCR reaction

1. Load the qPCR cyler and check PCR tubes and cyler lid for tight fit.
 2. Program the qPCR cyler or load an appropriate stored temperature profile. See Appendix for temperature profiles of selected qPCR cyclers. Programs for additional cyclers might be available on request. If parallel analysis (using the same cyler) of bacteria and fungi contaminations is required, the PCR program can be reduced to 40 cycles.
 3. Start the program and data reading.
-

8.5 Analysis

1. Save the data at the end of the run.
 2. Analyze the FAM™ and ROX™ channels and examine the obtained amplification plots.
 3. Set the threshold following the specific instructions provided in the manual of your cyler or those given in the Appendix, for selected devices. Check fluorescence data individually for typical amplification curves (logarithmic increase of fluorescence) and correct threshold settings manually, if needed.
 4. Analyze the calculation of the Ct values for negative controls, positive controls, and samples.
-

9. 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 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 (Ct ≥ 40)*	negative**	PCR inhibition
negative (Ct ≥ 40)*	positive (Ct < 40)	Mollicutes negative

* Samples showing Ct values ≥ 40 need to be evaluated individually. First, check their amplification curves for significant fluorescence increase in comparison to the background noise of the negative control. In case of substantial amplification, the curve should display the typical shape associated with a logarithmic “amplification” function. In general, we recommend repeating the testing of samples with such Ct values. In fact, even if a Ct ≥ 40 is not necessarily indicating amplification of mycoplasma DNA, it can reflect sample matrix effects caused by incorrect sample preparations or setup errors.

** If used as PCR control, the internal control of negative samples (FAM™ ≥ 40) must show Ct values in the range of ± 2 cycles (ROX™) of the negative control (master mix control, NTC). If used as process control, the internal control of negative samples (FAM™ ≥ 40) must show Ct values in the range of ± 3 cycles (ROX™) of the NTC.

10. Appendix

The protocol can be performed with any type of real-time PCR cycler able to detect the fluorescent dyes FAM™ and ROX™. The following qPCR cyclers were used for the validation of Microsart® ATMP Mycoplasma kit:

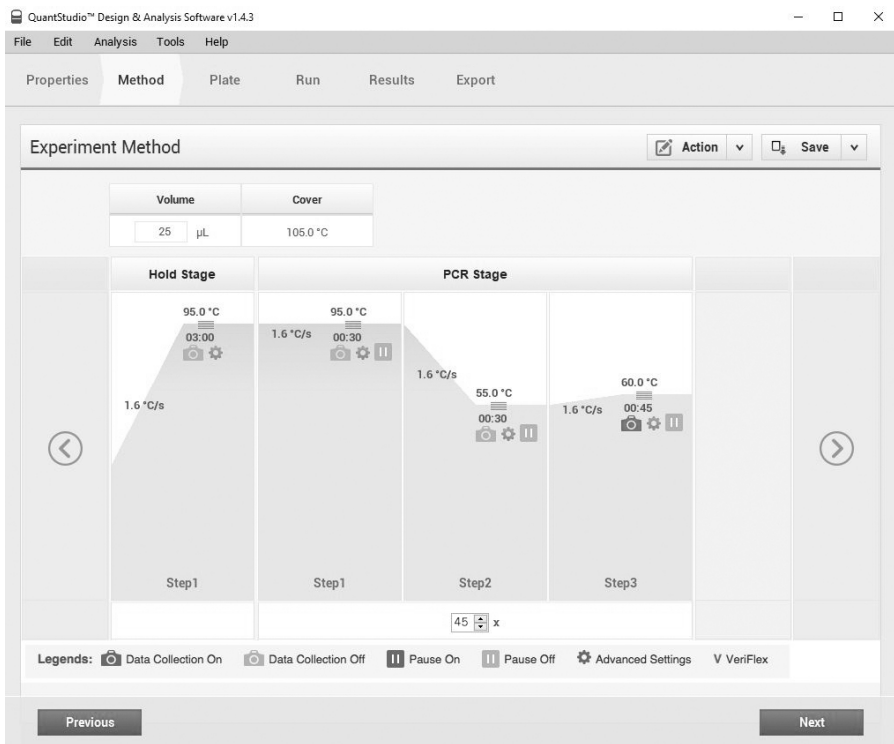
Rotor-Gene® (Qiagen), ABI Prism® 7500 (Applied Biosystems), Mx3005P® (Agilent Technologies), LightCycler® 1.0 and 2.0 (Roche).

In addition, Microsart® ATMP Mycoplasma kit was successfully tested with the following devices:

QuantStudio™, LightCycler® 480 II (Roche), CFX96 Touch™ / CFX96 Touch™ Deep Well (Bio-Rad), AriaMx™ (Agilent Technologies), StepOne™ / StepOne Plus™ (Applied Biosystems).

QuantStudio™

- Click File New Experiment Experiment Setup, to open the setup menu.
- Select Properties from the menu bar to open the Experiment Properties tab. In this tab, assign an experiment name (Name) and make sure that Chemistry is set to TaqMan® Reagents and that the Run mode option is set to Standard.
- Select Method in the menu bar to open the Experiment Method tab.
- Adjust the reaction volume and the cover temperature by setting Volume to 25 µl and Cover to 105 °C.
- Program a Hold Stage of 3 min at 95 °C.
- In PCR Stage, perform Step1 (denaturation step) at 95 °C for 30 sec, Step2 (annealing step) at 55 °C for 30 sec, and Step3 (elongation step) at 60 °C for 45 sec. Enable the data readout during the elongation step by clicking on the camera symbol. Set the number of cycles to 45. See also figure below for an overview of these settings.
- Select Plate in the menu bar to open the Assign Targets and Samples tab.
- Important: Set the Passive Reference to None!!!!
- Click on the Advanced Setup tab to define your targets and samples.
- In the Targets section: for the assay target, choose FAM™ as a reporter and NFQ-MGB as a quencher. For the internal control target, choose ROX™ as a reporter and NFQ-MGB as a quencher.
- Enter your samples names in the Samples section.
- Select the wells containing your samples (from the graphical overview of the plate), enable the targets, and assign the appropriate sample name by clicking on the respective check boxes.
- Select Run from the menu bar and click on START RUN to start the PCR run.



Data Analysis:

- To enable thresholds setting, please follow the specific instructions provided in the manual of your cycler.
- FAM™: adapt the threshold line to the initial linear section of the positive control reaction. ROX™: adapt the threshold line to the initial linear section of the NTCs.
- Select the Results tab to view specific Ct values.

Rotor-Gene® 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) 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 performed 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. FAM™: adapt the threshold line to the initial linear section of the positive control reaction. ROX™: adapt the threshold line to the initial linear section of the NTCs.
- 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. FAM™: adapt the threshold line to the initial linear section of the positive control reaction. ROX™: adapt the threshold line to the initial linear section of the NTCs.
- Samples showing no Ct value can be considered as negative.

Mx3005P™

- Go to the setup menu, click on Plate Setup, check all positions that 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

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, tap 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 automatic thresholds.
- To set the thresholds manually: for FAM™, adapt the threshold line to the initial linear section of the positive controls; for ROX™, adapt the threshold line to the initial linear section of the NTCs.
- Read the Ct values in Text Report.

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 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 [s]	60
Temperature Transition Rate [°C/s]	20.0
Secondary Target Temperature [°C]	0
Step Size [°C]	0
Step Delay [Cycles]	0
Acquisition Mode	None

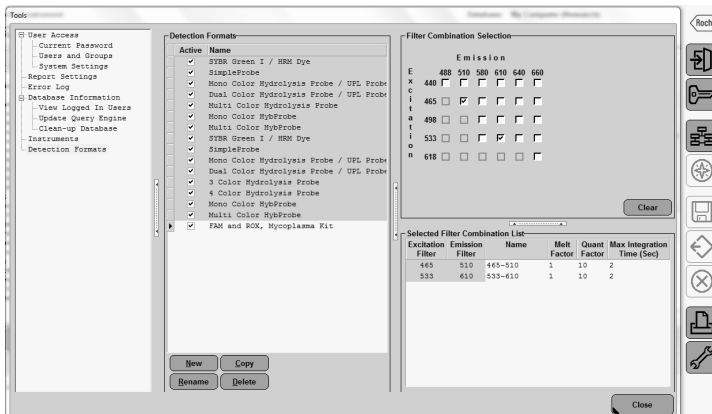
Analysis:

- 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.

LightCycler® 480 II

Choosing the correct filter setting:

- To define your filter combination go to the Tool menu at the lower right-hand corner.
- Click on Detection Formats on the left side and create a new detection format by clicking New.
- Give the new detection format a name, like FAM™ and ROX™ or Mycoplasma Kit.
- Select the right filter combination by clicking on the checkboxes with excitation 465 nm/ emission 510 nm (FAM™) and excitation 533 nm/emission 610 nm (ROX™).
- Choose following settings:
 - Melt Factor 1
 - Quant Factor 10
 - Max Integration Time (Sec) 2



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]	4.4
Secondary Target Temperature [°C]	0
Step Size [°C]	0.0
Step Delay [Cycles]	0
Acquisition Mode	None

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]	4.4	2.2	4.4
Secondary Target Temperature [°C]	0	0	0
Step Size [°C]	0.0	0.0	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]	2.2
Secondary Target Temperature [°C]	0
Step Size [°C]	0.0
Step Delay [Cycles]	0
Acquisition Mode	None

Data Analysis:

- Select the Threshold (Auto) to let the Software automatically adjust the threshold. This is the default setting.
- Please ensure that the automatically calculated threshold line is in the initial linear section of the reference sample (for FAM™: positive controls; for ROX™: NTCs). If not, switch to Log view and adapt the threshold line manually to the middle of the linear section of the reference sample. If you drag the threshold line, the software will set the threshold mode to Manual.
- Select the Results tab to view specific Ct values.

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

Run Setup Protocol Tab:

- Click File New Protocol to open the Protocol Editor and 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

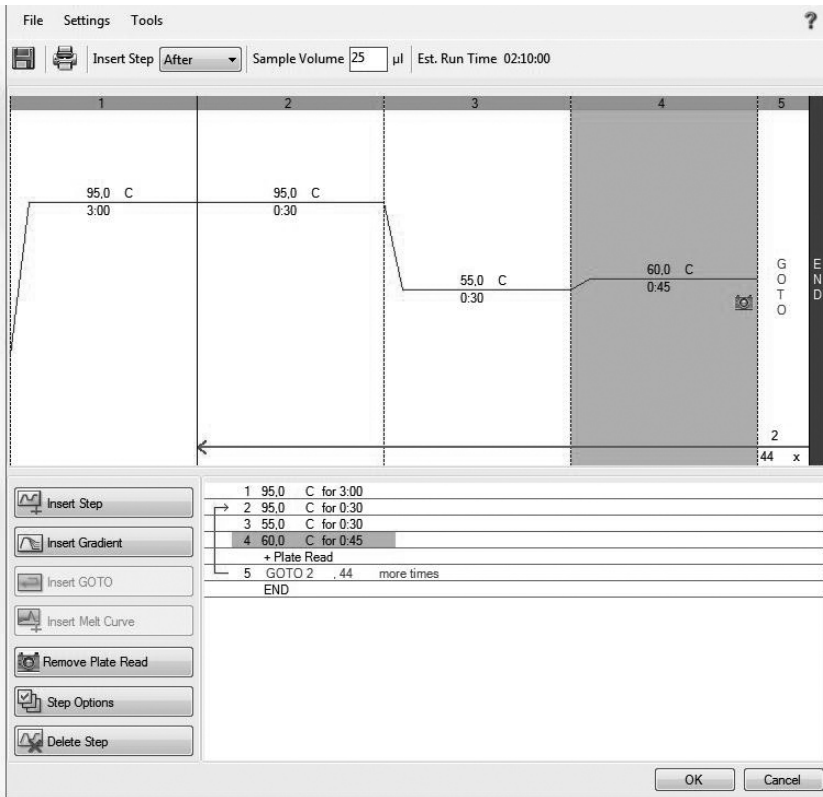
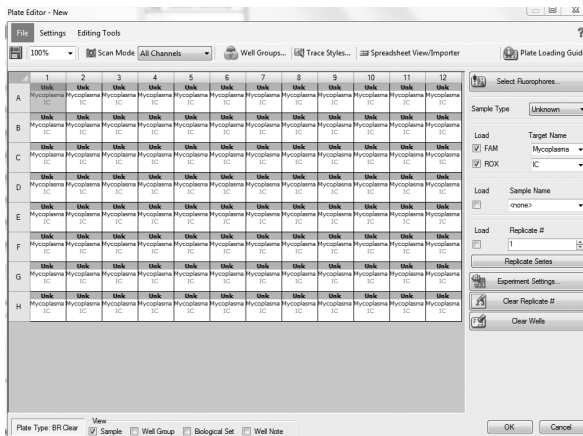


Plate Setup:

- Click File > New > Plate to open the Plate Editor and create a new plate.
- Specify the type of sample with Sample Type.
- Name your samples with 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.
- To enable thresholds setting, please follow the specific instructions provided in the manual of yourycler.
- FAM™: adapt the threshold line to the initial linear section of the positive controls.
- ROX™: adapt the threshold line to the initial linear section of the NTCs.
- Evaluate the Ct values according to chapter 9.

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™ to display data of mycoplasma detection and ROX™ 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.
- Please ensure that the automatically calculated threshold line is in the initial linear section of the reference sample (for FAM™: positive controls; for ROX™: NTCs). If not, 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 reference sample.
- Read specific Ct values at Result table.

StepOne™ / StepOne Plus™

Important information! In case the signal of the ROX™ channel is insufficient, please use 160 µl instead of 800 µl for rehydration of the internal control DNA (yellow cap).

- Go to the Experiment Properties tab under Setup and select under the type of experiment Quantitation – Standard Curve
- Select TaqMan Reagents to detect the target sequence.
- Select under ramp speed Standard.
- Go to Plate setup and click on the tab Define Targets and Samples.
- Select FAM™ as reporter to display data of mollicutes and ROX™ as reporter to display internal control amplification data.
- Select NFQ-MGB as Quencher for both reporters.

Experiment: 20160804-qPCR Myco Type: Standard Curve

Define Targets and Samples Assign Targets and Samples

Instructions: Define the targets to quantify and the samples to test in the reaction plate.

Define Targets

Add New Target Add Saved Target Save Target Delete Target

Target Name	Reporter	Quencher	Color
Mollicutes	FAM	NFQ-MGB	
Internal control	ROX	NFQ-MGB	

- Go to the Assign Target and Samples tab to view and edit the plate setup.
- Specify the type of samples and name your samples.
- Choose the fluorophore data you want to display by clicking the fluorophore checkboxes located under Assign targets to the selected wells. Select FAM™ (mollicutes) and ROX™ (internal control).
- Select None at the reference dye dropdown menu.
- Go to the tab Run Method and edit the temperature profile

Holding Stage:	1 cycle	Step 1:	3 min	95 °C	
Cycling Stage:	45 cycles	Step 2:	30 sec	95 °C	
		Step 3:	30 sec	55 °C	
		Step 4:	45 sec	60 °C	data collection
- Select 25 µL as Reaction Volume Per Well.
- Start run by clicking the green button.

Experiment Menu <<

Setup

Experiment Properties

Plate Setup

Run Method

Reaction Setup

Materials List

Run

Analysis

Experiment: 20100804-qPCR Myco

Define Targets and Samples **Assign Targets and Sa**

Instructions:
 To set up standards: Click "Define and Set Up Standards"
 To set up unknowns: Select wells, assign target(s), select "U"
 To set up negative controls: Select wells, assign target(s), the

Assign target(s) to the selected wells.

Assign	Target	Task	Quantity
<input checked="" type="checkbox"/>	Mollicules	[U] [S] [N]	
<input checked="" type="checkbox"/>	Internal control	[U] [S] [N]	

Mixed Unknown Standard Negative Control

Define and Set Up Standards

Assign sample(s) to the selected wells.

Assign	Sample
<input checked="" type="checkbox"/>	NEC
<input type="checkbox"/>	NTC
<input type="checkbox"/>	CP
<input type="checkbox"/>	M.F. 1

Assign sample(s) of selected well(s) to biological group.

Assign	Biological Group
	ROX
	FAM
	JOE
	NED
	SYBR
	TAMRA
	VIC
dye to use as the passive reference.	
	None
	None

Experiment: 20100804-qPCR Myco Type: Standard Curve

Run Method

Review the reaction volume and the thermal profile for the default run method. If needed, edit the default run method or select a run method from the

Graphical View Tabular View

Reaction Volume Per Well: 25 µL

Legend: Data Collection On, Data Collection Off, AutoData On, AutoData Off, Zone Temperatures

Data Analysis:

- Go to the Analysis tab and click Amplification Plot.
- Under Plate Settings and the Plot Type dropdown menu select ΔRn vs Cycle.
- Select linear graph type and click the checkboxes Auto Baseline and Auto Threshold
- Please ensure that the automatically calculated threshold line is in the initial linear section of the reference sample (for FAM™: positive controls; for ROX™: NTCs). If not, switch to Log view and adapt the threshold line manually to the middle of the linear section of the reference sample. If this is not the case change to Log view and adapt the threshold line manually.
- Change the Plate Layout to Well Table and see the specific Ct values.

11. Related Products

Detection Kits for qPCR

SMB95-1001/1002	Microsart® AMP Mycoplasma	25/100 tests
SMB95-1005/1006	Microsart® RESEARCH Mycoplasma	25/100 tests
SMB95-1007	Microsart® ATMP Sterile Release	10 samples
SMB95-1008	Microsart® ATMP Bacteria	100 tests
SMB95-1009	Microsart® RESEARCH Bacteria	25 tests
SMB95-1012	Microsart® ATMP Fungi	100 tests
SMB95-1014/1013	Microsart® RESEARCH Fungi	25/100 tests

Microsart® Calibration Reagent, 10⁸ genomes / vial, 1 vial (bacteria, including Mollicutes)

SMB95-2021	<i>Mycoplasma arginini</i>
SMB95-2022	<i>Mycoplasma orale</i>
SMB95-2023	<i>Mycoplasma gallisepticum</i>
SMB95-2024	<i>Mycoplasma pneumoniae</i>
SMB95-2025	<i>Mycoplasma synoviae</i>
SMB95-2026	<i>Mycoplasma fermentans</i>
SMB95-2027	<i>Mycoplasma hyorhinis</i>
SMB95-2028	<i>Acholeplasma laidlawii</i>
SMB95-2029	<i>Spiroplasma citri</i>
SMB95-2030	<i>Bacillus subtilis</i>
SMB95-2031	<i>Pseudomonas aeruginosa</i>
SMB95-2032	<i>Kocuria rhizophila</i>
SMB95-2033	<i>Clostridium sporogenes</i>
SMB95-2034	<i>Bacteroides vulgatus</i>
SMB95-2035	<i>Staphylococcus aureus</i>
SMB95-2036	<i>Mycoplasma salivarium</i>

Microsart® Calibration Reagent, 10⁶ genomes / vial, 1 vial (fungi)

SMB95-2044	<i>Candida albicans</i>
SMB95-2045	<i>Aspergillus brasiliensis</i>
SMB95-2046	<i>Aspergillus fumigatus</i>
SMB95-2047	<i>Penicillium chrysogenum</i>
SMB95-2048	<i>Candida glabrata</i>
SMB95-2049	<i>Candida krusei</i>
SMB95-2050	<i>Candida tropicalis</i>

Microsart® Validation Standard, 10 CFU / vial, 3 vials each (Mollicutes)

SMB95-2011	<i>Mycoplasma arginini</i>
SMB95-2012	<i>Mycoplasma orale</i>
SMB95-2013	<i>Mycoplasma gallisepticum</i>
SMB95-2014	<i>Mycoplasma pneumoniae</i>
SMB95-2015	<i>Mycoplasma synoviae</i>
SMB95-2016	<i>Mycoplasma fermentans</i>
SMB95-2017	<i>Mycoplasma hyorhinis</i>
SMB95-2018	<i>Acholeplasma laidlawii</i>

SMB95-2019 Spiroplasma citri
 SMB95-2020 Mycoplasma salivarium

Microsart® Validation Standard, 100 CFU / vial, 3 vials each (Mollicutes)

SMB95-2051 Mycoplasma orale
 SMB95-2052 Mycoplasma pneumoniae

Microsart® Validation Standard, 99 CFU / vial, 6 vials each (bacteria* and fungi)

SMB95-2005 Bacillus subtilis
 SMB95-2006 Pseudomonas aeruginosa
 SMB95-2007 Kocuria rhizophila
 SMB95-2008 Clostridium sporogenes
 SMB95-2009 Bacteroides vulgatus
 SMB95-2010 Staphylococcus aureus
 SMB95-2037 Candida albicans
 SMB95-2038 Aspergillus brasiliensis
 SMB95-2039 Aspergillus fumigatus
 SMB95-2040 Penicillium chrysogenum
 SMB95-2041 Candida glabrata
 SMB95-2042 Candida krusei
 SMB95-2043 Candida tropicalis

* except for Mollicutes

DNA Extraction Kit

SMB95-2001	Microsart® ATMP Extraction (for bacteria and fungi)	50 extractions
SMB95-2003	Microsart® AMP Extraction (for mycoplasma)	50 extractions
SMB95-2002	Microsart® AMP Coating Buffer	20 × 2 ml
56-0002	Proteinase K**	50 extractions

PCR Clean™ **

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

PCR Clean™ Wipes**

15-2001	DNA Decontamination Reagent, Wipes	50 wipes
15-2002	DNA Decontamination Reagent, refill sachets	5 × 50 wipes

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Last technical revision: 2020-03-30

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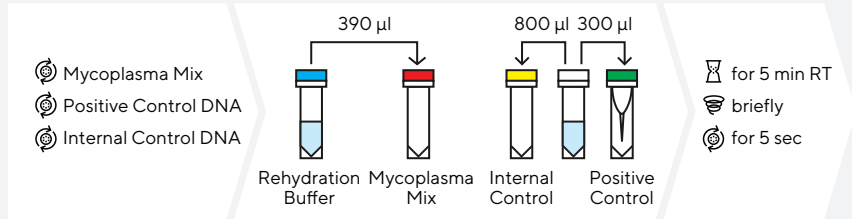
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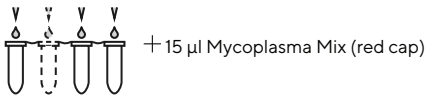
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1. Rehydration of Reagents



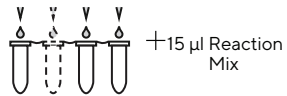
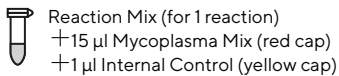
2. Preparation of PCR Reaction Mix

a) Internal Control added during DNA extraction

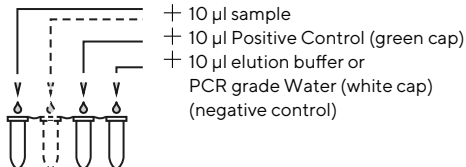


* do not forget to add 1 µl Internal Control to NTC and PC

b) Internal Control not added during DNA extraction

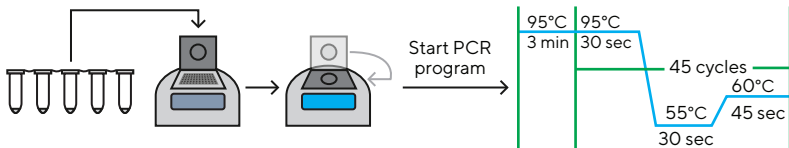


3. Addition of Samples and Controls



Close lid tightly
 🌀 briefly

4. Start of the qPCR Reaction



- Rehydration Buffer
- Mycoplasma Mix
- PCR grade Water
- Positive Control
- Internal Control

- ⏰ 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®-ATMP-Mycoplasma_03_EN

Short Instructions

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Order No.: 85037-546-76

Ver. 12 | 2021