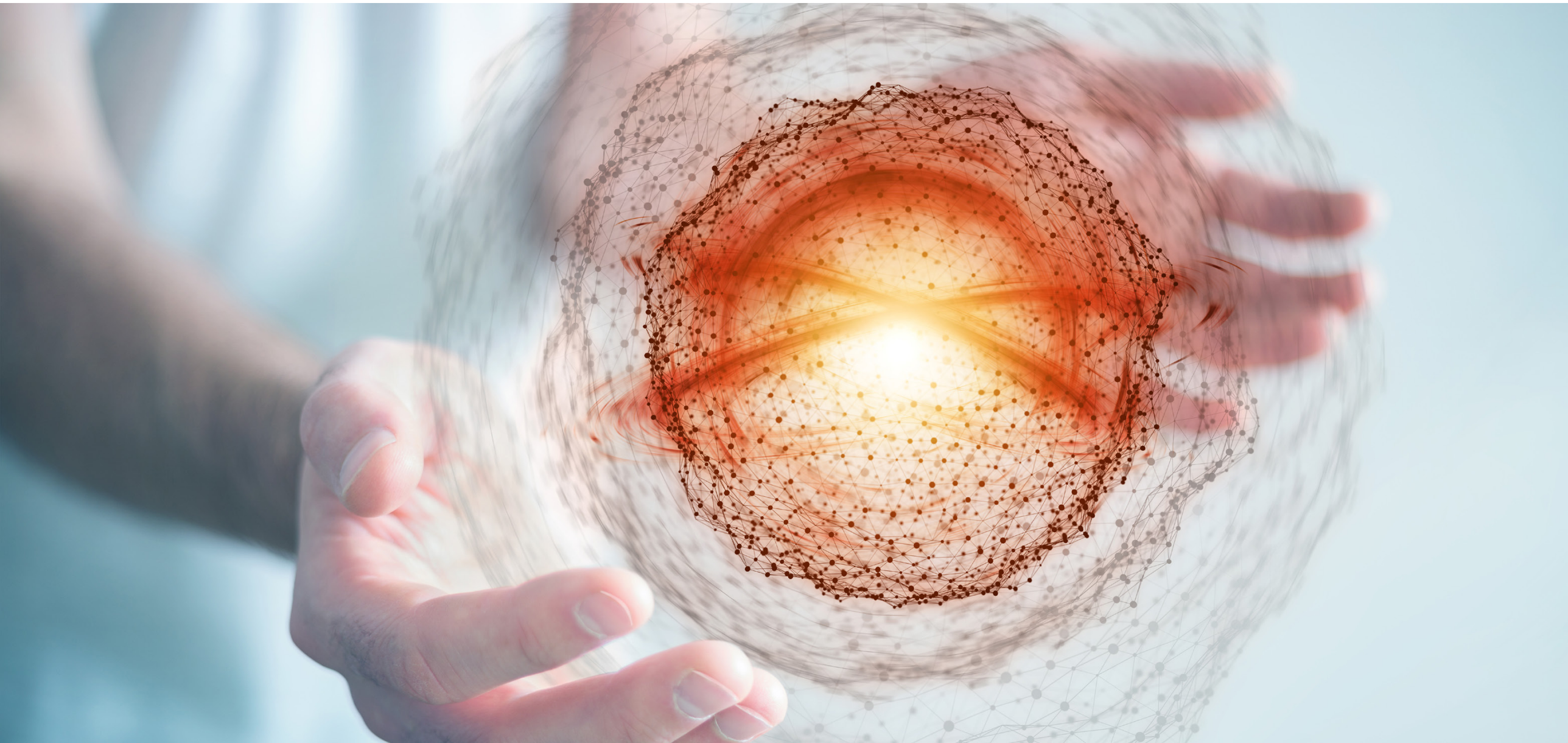
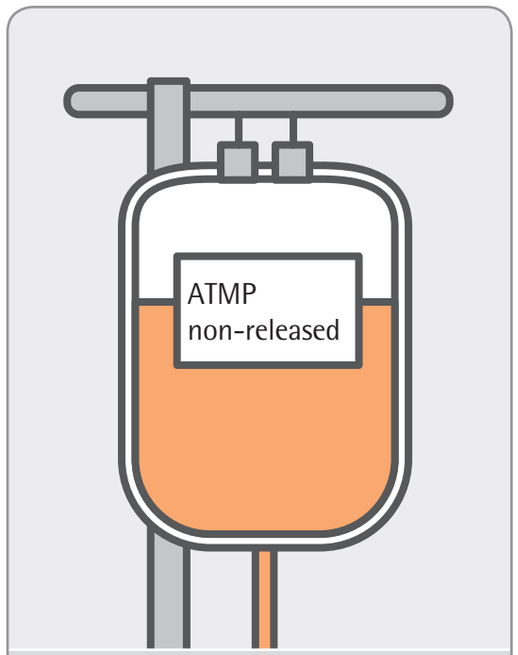
Cell-Based Therapies – Be on the Safe Side

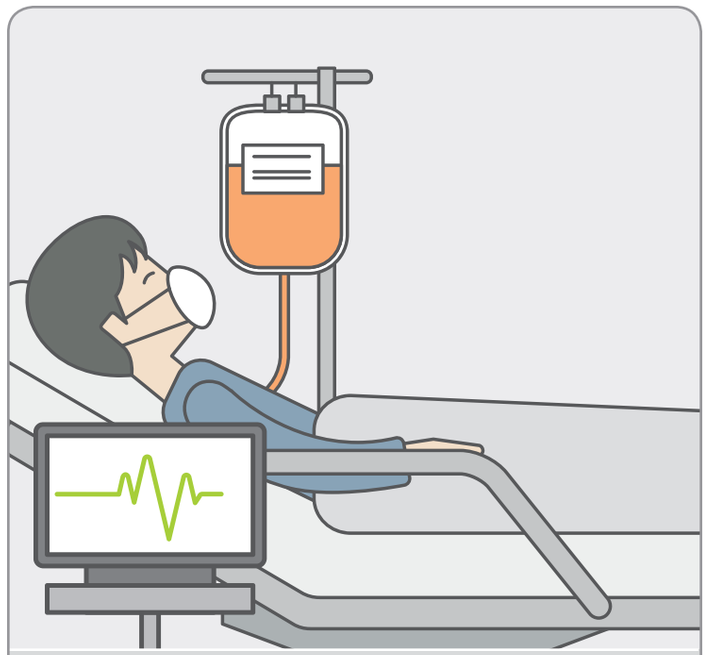
Get QC Results for Advanced Therapy Medicinal Products (ATMPs) Before Treatment



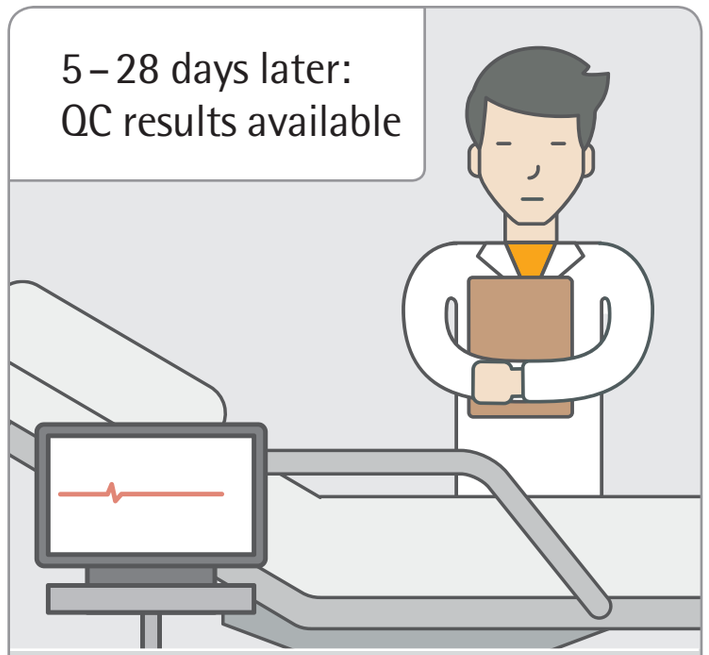
The Safety of the Patient is at Risk



QC result pending



Treatment despite risk:
ATMP might be contaminated



"Sorry, the ATMP
was contaminated."

- The Challenge: Growth-based QC-release takes too long. How do you ensure non-contaminated ATMPs?
- Need: Short-shelf life ATMPs need rapid QC-results.

Click here for details of critical QC-release time line

Obtain On-Time QC Results for the Patient's Safety within 3 Hours

Microsart® ATMP Mycoplasma

Microsart® ATMP Bacteria

qPCR based method

Obtain On-Time QC Results for the Patient's Safety within 3 Hours

Microsart® ATMP Mycoplasma

qPCR method

§ Compliant testing

Microsart® ATMP Bacteria

§ Detect >95% of known Bacteria

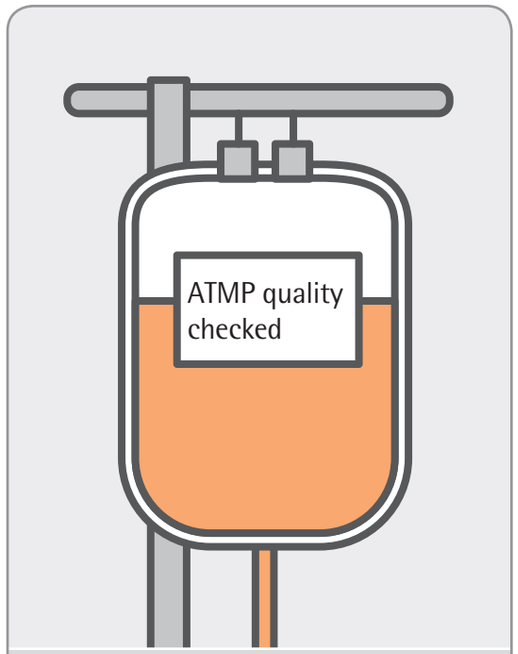
Minimize false-negatives

Deliver fewer false-positives

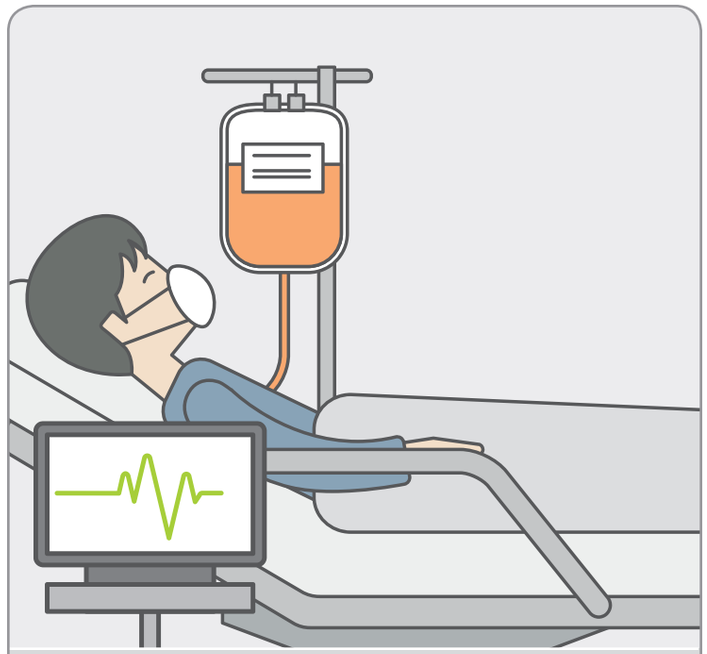
Avoid cross-contamination

Straightforward in-house validation

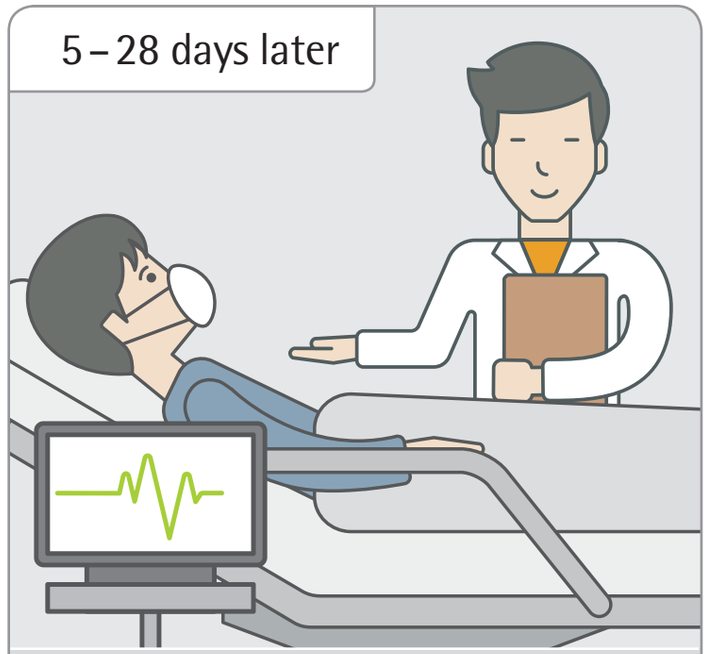
Help Ensure Your Patient's Safety



QC result available



Treatment with checked quality



"Your prospects for recovery look promising."

Result: You can control the Quality of your ATMP before a patient is treated.

Click here for details of in-time QC-results before treatment

Help Ensure Your Patient's Safety

- Obtain Quality Control Results prior treatment
- Validated method
- Avoid cross contamination by using inactivated, non-infectious validation standards
- Minimize false-negatives
- Save 75% in handling and analysis due to included inhibition control
- Profit by our professional validation guidance

Coming in 2019: Microsart® ATMP Fungi
Contact: PCR@Sartorius.com

Our dedicated Regenerative Medicine group will support you with our comprehensive portfolio for ATMP development and production.
Just contact your Sartorius sales represenative.

Microsart® ATMP Mycoplasma

See attached file
Datasheet Microsart® ATMP Mycoplasma

See attached file
Flyer Mycoplasma Assays for qPCR – The new Gold Standard

See attached file
Manual Microsart® ATMP Mycoplasma incl. pipetting sheme

See attached file
Application Note Microsart® AMP Mycoplasma: Benchmark

See attached file
Application Note Microsart® AMP Mycoplasma GC | CFU ratio

Microsart® ATMP Bacteria

See attached file
Datasheet Microsart® ATMP Bacteria

See attached file
Manual Microsart® ATMP Bacteria

See attached file
Manual Microsart® Bacteria EXTRACTION

See attached file
Manual Microsart® Bacteria Patient

See attached file
Manual Microsart® Calibration Reagent

See attached file
Manual Microsart® Research Bacteria

See attached file
Manual Microsart® Validation Standard Bacteria

See attached file
Application Note Comparison Microsart ATMP Bacteria

See attached file
Application Note Beta Test: Real-time PCR

Visit us for more information:
https://promotions.sartorius.com/real-heroes/

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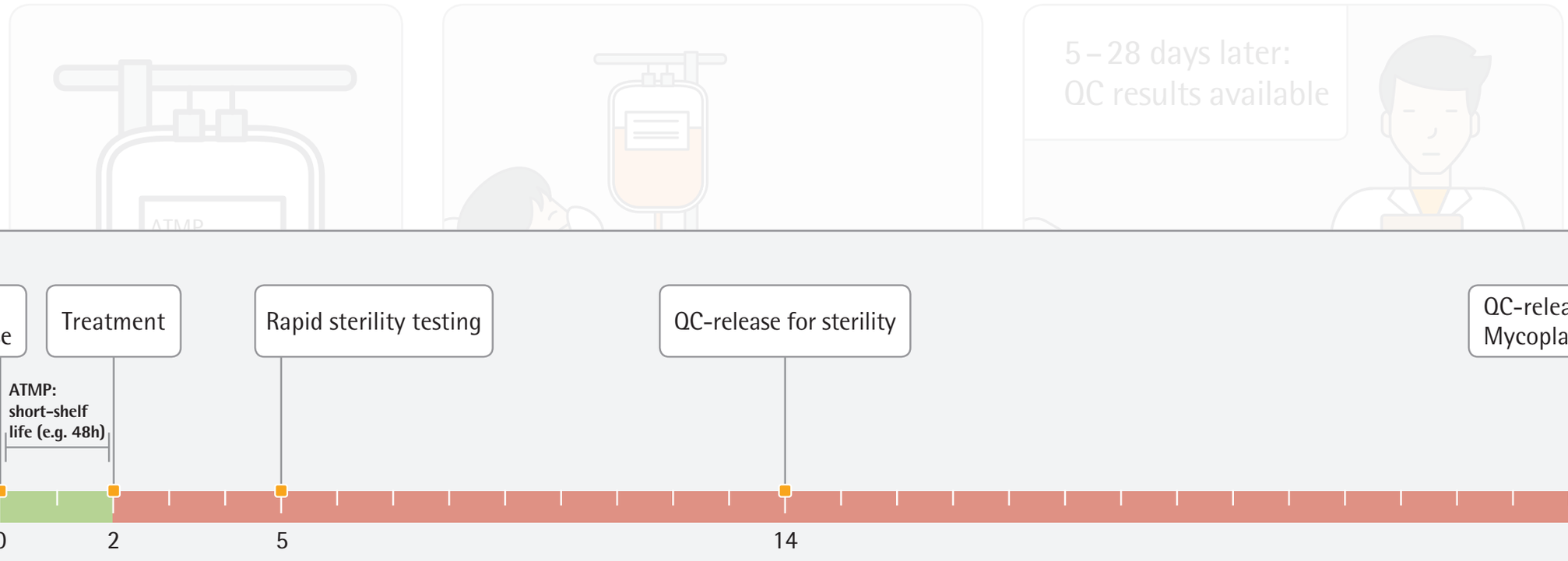
The Safety of the Patient is at Risk



- The Challenge: Growth-based QC-release takes too long. How do you ensure non-contaminated ATMPs?

- Need: Short-shelf life ATMPs need rapid QC-results.

Obtain On-Time QC Results for the Patient's Safety within 3 Hours

Microsart® ATMP Mycoplasma

qPCR method

§ Compliant testing as the kits have been validated according to EP 2.6.7. for compendial product release.

Detailed Validation Report available on request.

Please contact:
PCR@Sartorius.com

Microsart® ATMP Bacteria

§ Detect >95% of known Bacteria

iver fewer
e-positives

Avoid cross-contamination

Straightforward in-house validation

Obtain On-Time QC Results for the Patient's Safety within 3 Hours

Microsart® ATMP Mycoplasma

qPCR method

§ Compliant testing

Minimize false-negatives

Deliver fewer false-positives

A
c

Microsart® ATMP Bacteria

§

Detect >95% of known Bacteria

Current guideline status:
Kit is not suitable yet to replace classical sterility testing according to EP 2.6.1. and USP<71>

Validated according to EP 5.1.6. | USP<1223> | EP 2.6.27 | TR33 for alternative methods

Sensitivity (5 – 99 CFU/mL) proven for 18 selected bacterial species including 6 standard USP | EP strains AND 12 critical cell therapy contaminants that are difficult to detect by classical sterility testing, but can be detected specifically and robust by qPCR.

Obtain On-Time QC Results for the Patient's Safety within 3 Hours

Microsart® ATMP Mycoplasma

qPCR method

Microsart® ATMP Bacteria

qPCR method

Kits can be used with almost all types of qPCR cycler able to detect FAM™ & ROX™.

Both kits can be used in the same qPCR run simultaneously.

Obtain On-Time QC Results for the Patient's Safety within 3 Hours

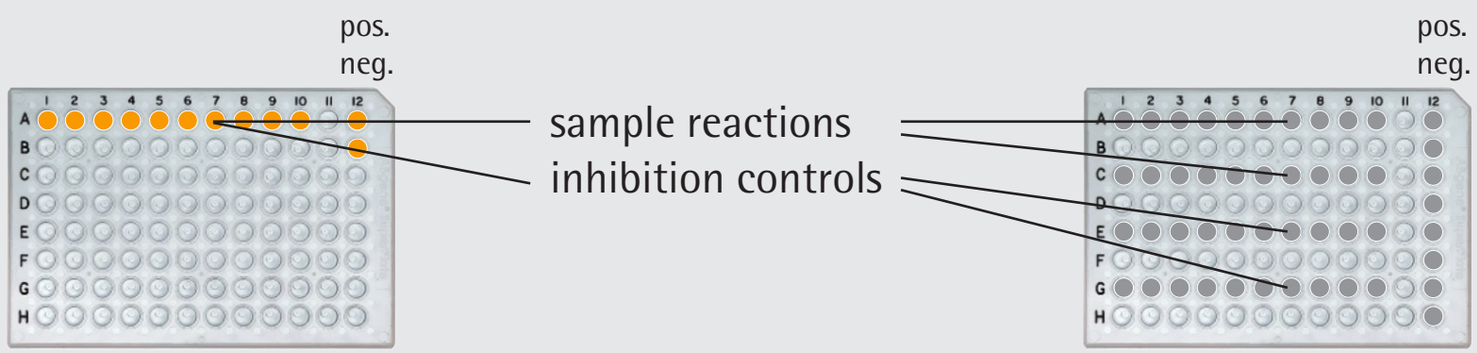
Microsart® ATMP Mycoplasma

qPCR method

Microsart® ATMP Bacteria

Minimize false-negatives. Save 75% in handling and analysis.

Obtain result for Sample and Inhibition control from a single well (Multiplex PCR).
Example: Testing 10 samples for Mycoplasma contamination.



Sartorius: 12 tubes

Vendor B: 48 tubes

Obtain On-Time QC Results for the Patient's Safety within 3 Hours

Microsart® ATMP Mycoplasma

qPCR method

Microsart® ATMP Bacteria

Deliver fewer false-positives by use of highly specific TaqMan™ probes instead of unspecific SYBR-Green dye

See attached file
Application Note Microsart® AMP Mycoplasma: Benchmark

Obtain On-Time QC Results for the Patient's Safety within 3 Hours

Microsart® ATMP Mycoplasma

qPCR method

Microsart® ATMP Bacteria

Keep your facility clean and avoid cross-contamination by using inactivated, non-infectious validation standards (ready-to-use in 10 CFU/mL)

See attached file
Application Note Microsart® AMP Mycoplasma GC | CFU ratio

Obtain On-Time QC Results for the Patient's Safety within 3 Hours

Microsart® ATMP Mycoplasma

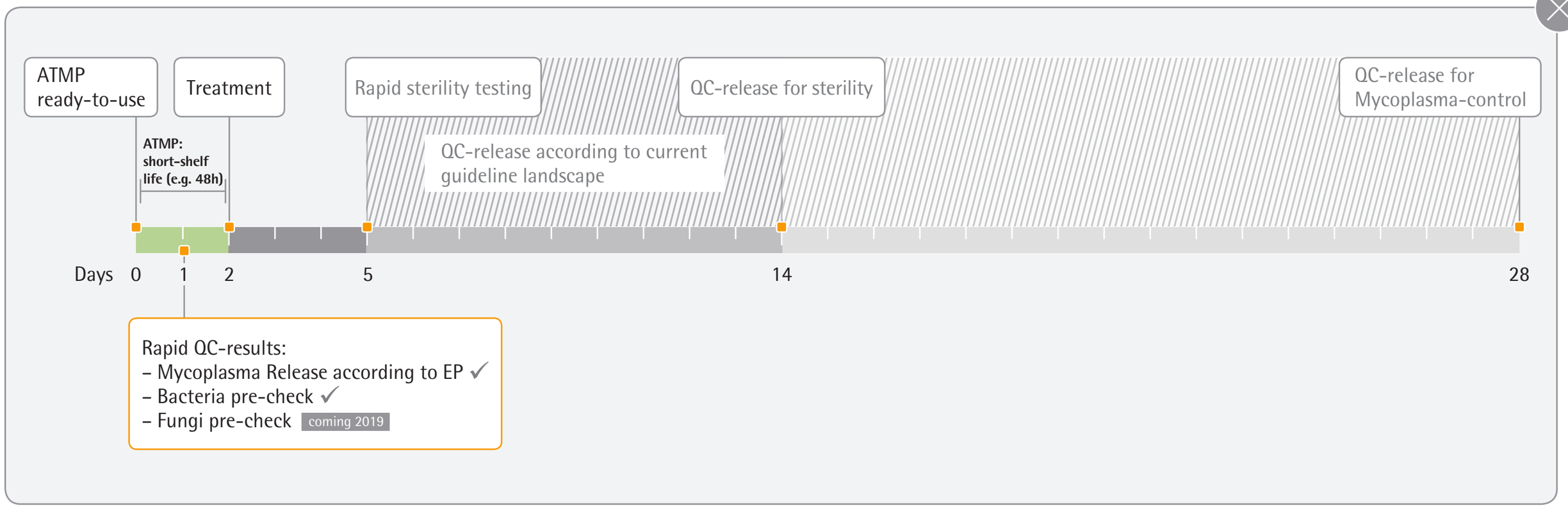
qPCR method

Microsart® ATMP Bacteria

Straightforward in-house validation with detailed step-by-step guidance by Sartorius qPCR experts.

Please contact: **PCR@Sartorius.com**

Help Ensure Your Patient's Safety



Result: You can control the Quality of your ATMP before a patient is treated.

Microsart® ATMP Mycoplasma

Rapid Real-time PCR Mycoplasma Detection Kit for testing ATMPs

User Benefits

- Highest Level of Security
- Designed for ATMP Testing
- Easy Handling

Product Information

A standard DNA extraction followed by a TaqMan® probe real-time qPCR is used for the detection of Mycoplasma DNA. 200 µl sample volume can be used as starting material for DNA preparation. The isolated DNA is amplified in a qPCR cycler and the evaluation can be performed with the standard cycler software.

Introduction

Microsart® ATMP Mycoplasma utilizes quantitative, real-time PCR (qPCR) as the method of choice for sensitive and reliable detection of Mycoplasma contamination in autologous cell transplant culture. The Microsart® ATMP Mycoplasma kit was validated according EP 2.6.7 in combination with EP 2.6.21 with respect to detection limit for all listed Mycoplasma species, specificity and robustness for autologous cell transplants (e.g. chondrocytes).

Applications

The Microsart® ATMP Mycoplasma real-time PCR kit is especially designed for all hospitals, institution and companies which are involved in testing Mycoplasma contamination according to EP 2.6.7 in cell-based therapeutics like autologous chondrocyte transplants (ATMPs – advanced therapy medical products).

High Performance

The Microsart® ATMP Mycoplasma kit has been developed for EP complaint Mycoplasma testing in autologous cell transplants. A detection limit of less than 10 cfu/ml for all Mycoplasma species mentioned in the European Pharmacopoeia fulfills the requirements for the needed sensitivity and specificity.

Fast Result

The Microsart® ATMP Mycoplasma kit is a fast and easy to use real-time PCR kit. The total procedure from DNA extraction to PCR result takes only a few hours.

TaqMan® Probes

The use of TaqMan® probes adds specificity to the PCR detection system. The analysis is performed during the cycling process – no melting curve analysis is needed.

Contamination Prevention

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

Summary

The Microsart® ATMP Mycoplasma kit is the optimal solution for all QC labs which performing Mycoplasma testing of cell-based therapeutics like autologous chondrocyte transplants.

Unlike competitive PCR Detection kits, the Microsart® ATMP Mycoplasma kit is dedicated for the specific application in regard to sample volume, sensitivity , robustness and specificity.

Technical Specifications

Each Kit contains all required reagents for 25 or 100 reactions including polymerase as part of the Mycoplasma Mix. The expiry date of the unopened package is specified on the package label. The kit components are to be stored until use at +2 to +8°C and must be stored after opening and rehydration below –18°C. The lot specific Certificate of Analysis can be downloaded from the manufacturer's website (www.minerva-biolabs.com).

Kit Component	25 Reactions	100 Reactions
Order No.	SMB95-1003	SMB95-1004
Mycoplasma Mix	1 × lyophilized	4 × lyophilized
Rehydration Buffer	1 × 1.0 ml	4 × 0.5 ml
Positive Control	1 × lyophilized	1 × lyophilized
Internal Control	1 × lyophilized	4 × lyophilized
PCR grade Water	1 × 1.5 ml	4 × 1.5 ml

Order Information

Description	Quantity	Order No.
Mycoplasma Kits		
Microsart® ATMP Mycoplasma	25	SMB95-1003
Microsart® ATMP Mycoplasma	100	SMB95-1004
Accessories		
Microsart® AMP Extraktion	50 extractions	SMB95-2003
Related Products		
Microsart® AMP Mycoplasma	25	SMB95-1001
Microsart® AMP Mycoplasma	100	SMB95-1002
Microsart® RESEARCH Mycoplasma	25	SMB95-1005
Microsart® RESEARCH Mycoplasma	100	SMB95-1006

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Russian Federation +7.812.327.53.27
Japan +81.3.3740.5408

Publication No.: SM-2012-e171004
Order No.: 85037-540-16
Ver. 10 | 2017

Instructions for Use

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
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® ATMP Mycoplasma is used for the direct detection of Mollicutes (*Mycoplasma*, *Acholeplasma*, *Spiroplasma*) in cell cultures and cell culture derived biologicals, like autologous transplants (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 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 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 25 reactions Order No. SMB95-1003	 100 reactions Order No. SMB95-1004	Cap Color
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

5. 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
- 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)
  Microsart® Validation Standard available for all EP-listed mycoplasma species (see page 34)
  Microsart® Calibration Reagent available for all EP-listed mycoplasma species (see page 34)
  Uracil-N-glycosylase (see page 34)
  10 mM Tris buffer, pH 8.4

6. Specimen

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 (see 6.3).

Concentration step for sample volumes > 200 µl:

For cell culture or cell culture supernatants with volumes of 200 to 1000 µl 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. The sample can contain up to 10^6 cells.

1.	Transfer 1 ml of cell culture or cell culture supernatant into a sterile reaction tube.
2.	Centrifuge the sample at 10,000 × g for 15 minutes to sediment mycoplasma particles. Alternatively: centrifuge the sample for 6 minutes at 13,000 × g.
3.	Discard the supernatant and suspend the pellet in 200 µl buffer (10 mM Tris, pH 8.4).
4.	Vortex the sample and use for DNA extraction.

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 used should be suitable 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 10 CFU mycoplasma by using special reference materials (Microsart® Validation Standards, see page 35) 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 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 should be avoided and reconstituted controls (Internal Control DNA and Positive Control DNA) 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 micro centrifuge
2.	Mycoplasma Mix	red cap	Add 390 µl Rehydration Buffer (blue cap)
3.	Internal Control DNA	yellow cap	Add 800 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 fluorescent 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	15.0 µl	375.0 µl
Internal Control DNA	yellow	1.0 µl	25.0 µl

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

3. Add 15 µl to each PCR tube. Discard remaining liquid.

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

8.3 Loading the test tubes

1. Negative controls: add 10 µl elution buffer from DNA extraction kit 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).
4. Close tightly and spin all PCR tubes briefly.

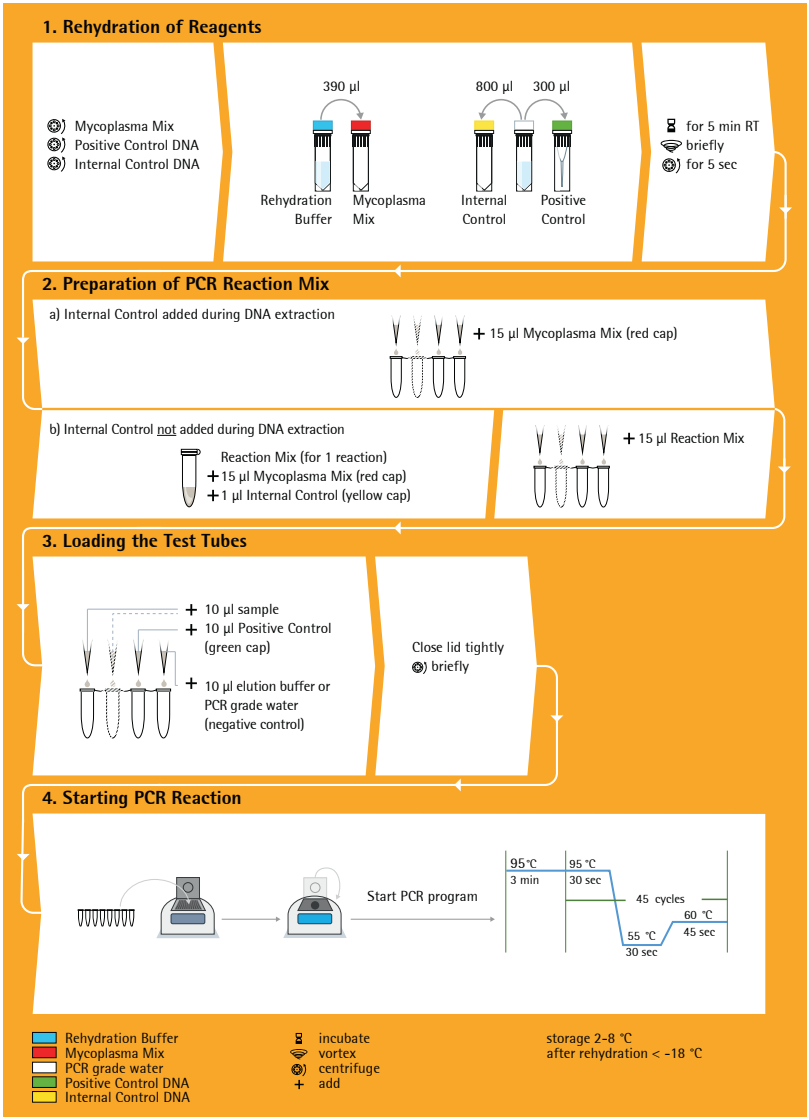
8.4 Starting the reaction

1. Load the qPCR cycler and check each PCR tube and the cycler lid for tight fit.
2. Program 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.

8.5 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 all 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 negative controls, positive controls and samples.

9. Short Instructions



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

ST_SI_Microsart®-ATMP-Mycoplasma_01_EN

10. Notes on the Test Procedure

1. This leaflet must be widely understood for a successful use of 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.

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 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® Validation Standards or Microsart® Calibration Reagents, available for all mycoplasma species listed in EP 2.6.7 (see page 34).
   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® ATMP Mycoplasma kit:

Rotorgene® 6000 (5-plex)
ABI Prism® 7500
Mx3005P®

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

LightCycler® 1.0 and 2.0
LightCycler® 480 II
CFX96 Touch™ / CFX96 Touch™ Deep Well
AriaMx
ABI StepOne™ / StepOne Plus™

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*

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

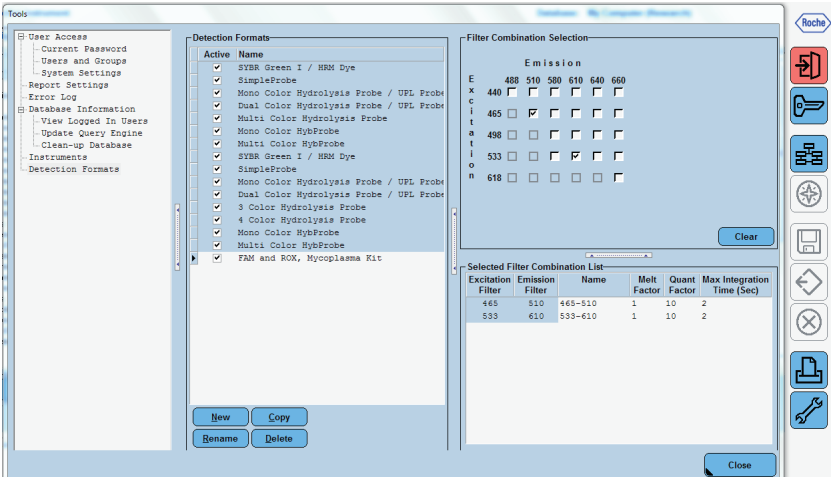
Analysis:
- Select the fluorescence channels *Channel 1* (520 nm) and *Channel 3* (610 nm)
- Click on *Quantification* to generate the amplification plots and the specific Ct-values
- The threshold will be generated automatically

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 the checkboxes with an 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]	10	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
- The automatic threshold line should be adapted to the initial linear section of positive control. If this is not the case change to Log view and adapt the threshold line manually to the middle of the linear section of the positive control. If you drag the threshold line the software automatically sets 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 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		

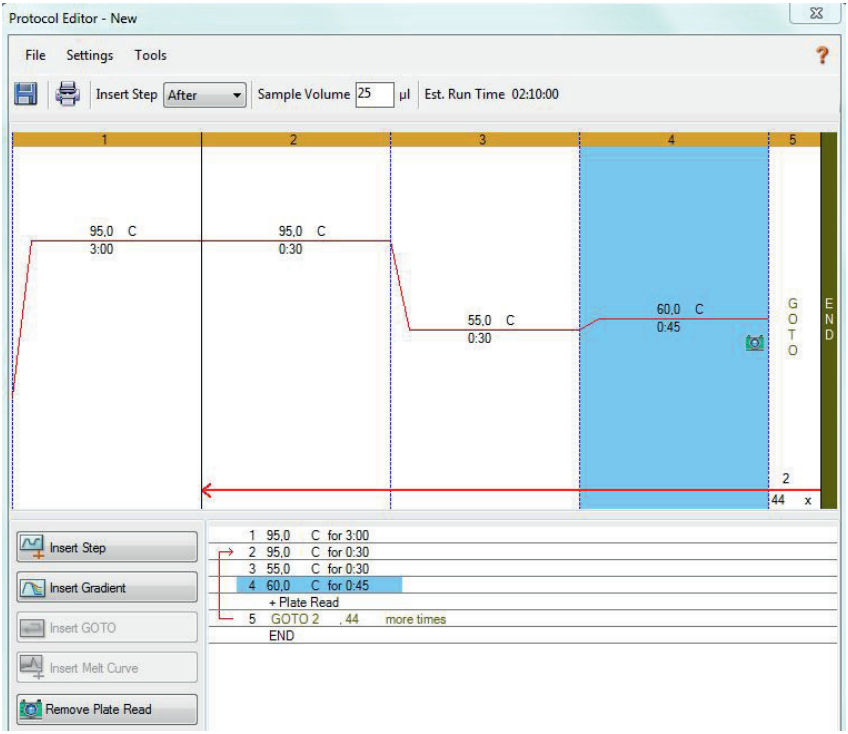
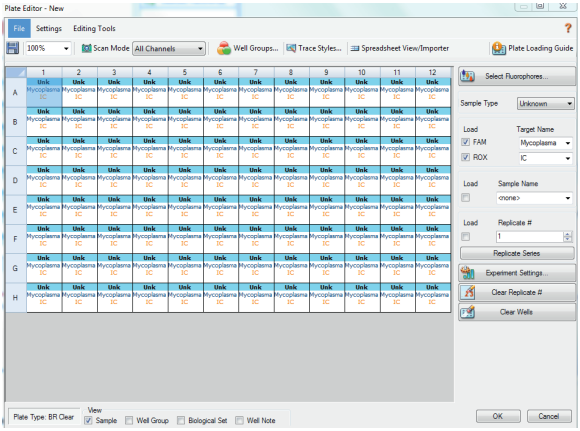


Plate Setup:

- 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:

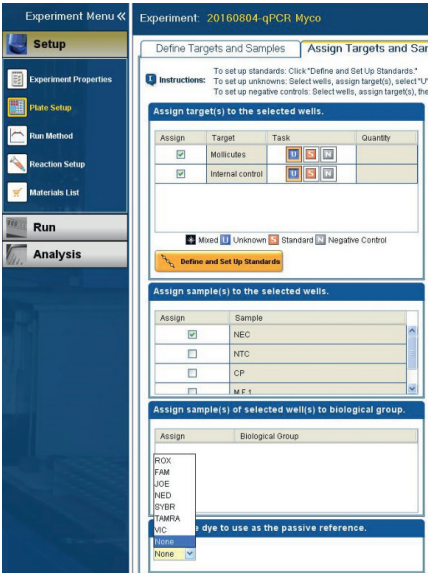
- In the area *Analysis* click on *Analysis Criteria* and mark the wells you want to analyze
- By clicking the checkbox at the top left-hand of the plate screen you can select all wells
- By changing to *Graphical Display* tab you can see the amplification plot
- Choose *ΔR* (baseline corrective raw 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*

ABI 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™ (blue) as reporter to display data of mollicutes and ROX (green) as reporter to display internal control amplification data
- Select NFQ-MGB as Quencher for both reporters

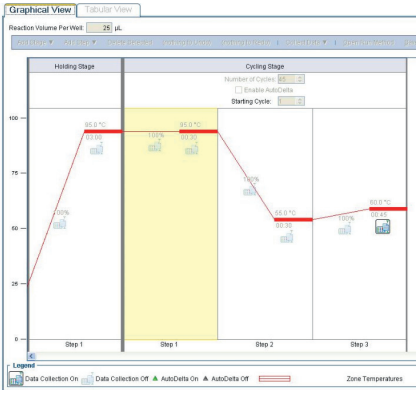
- Go to the *Assign Target and Samples* tab where you can view and edit the plate setup
- Specify the type of samples and name your samples
- Choose the fluorophore data you want to display by clicking the fluorophore checkboxes located under *Assign targets to the selected wells*. Select FAM™ (mollicutes) 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



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*
- The automatic threshold line should be adapted to the initial linear section of positive control. If this is not the case change to Log view and adapt the threshold line manually to the middle of the linear section of the positive control
- Change the *Plate Layout* to *Well Table* and see the 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. Minerva Biolabs shall have no liability for any direct, indirect, consequential, or incidental damages arising from the use, the results of use, or the inability to use this product.

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Last technical revision: 2017-06

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/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

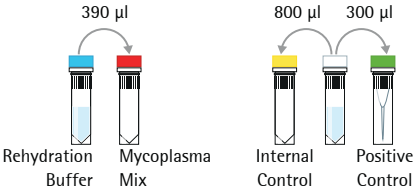
UNG Carry over prevention*

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

*** Distributed by Minerva Biolabs**

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

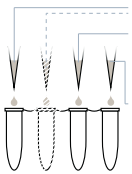
+ 15 µl Mycoplasma Mix (red cap)

b) Internal Control not added during DNA extraction

Reaction Mix (for 1 reaction)
+ 15 µl Mycoplasma Mix (red cap)
+ 1 µl Internal Control (yellow cap)

+ 15 µl Reaction Mix

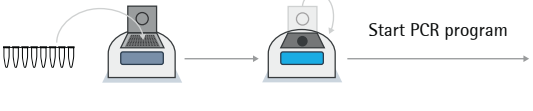
3. Loading the Test Tubes



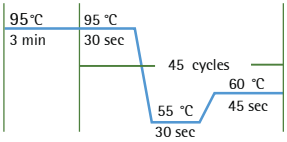
+ 10 µl sample
+ 10 µl Positive Control (green cap)
+ 10 µl elution buffer or PCR grade water (negative control)

Close lid tightly
briefly

4. Starting PCR Reaction



Start PCR program



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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37079 Goettingen, Germany

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Fax +49.551.308.3289
www.sartorius-stedim.com

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specifications and illustrations
in this manual is indicated by
the date given below.
Sartorius Stedim Biotech GmbH
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specifications and design of the
equipment without notice.

Status:
June 2016,
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Goettingen, Germany

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has been bleached without any use
of chlorine. | W
Publication No.: SM-6111-e150104
Order No.: 85037-546-76
Ver.02 | 2017

Benchmarking of the Microsart® AMP Mycoplasma kit with products of two other suppliers

Application Note

#09
#10
#11
#12
#13

turning science into solutions

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Introduction

Among the world's smallest bacteria Mycoplasmas are capable of independent reproduction. They belong to the class of Mollicutes and have a very slow and parasitic growth. The contamination of cell cultures remains a major problem. An array of physiological and biochemical parameters are affected by the presence of Mycoplasma in cells cultures. The infection causes changes in metabolism, growth, viability, macromolecule synthesis, morphology etc. and therefore a sensitive routine testing for contamination of cell cultures is essential.

The traditional growth-based method requires a cultivation time of at least 28 days before a contamination can be ruled out with certainty. In comparison, Nucleic Acid Amplification Techniques (NAT) allow to reduce time to results to just hours. As an alternative to the culture method the NAT test system must be shown to detect 10 CFU/mL of Mycoplasma. For this reason the capability of three different Mycoplasma PCR kits to detect spikes of not higher than 10 CFU per ml in Dulbecco's Modified Eagle Medium was tested in this study.

Experimental Set-up and Results

During this study the Microsart® AMP Mycoplasma kit inclusive sample preparation with the kit Microsart® AMP Extraction (Sartorius Stedim Biotech GmbH) were benchmarked with two other Mycoplasma detection kits which are also based on DNA isolation with subsequent Real-time PCR analysis. As test material the 10CFU Sensitivity Standards *Mycoplasma arginini* (NCTC code 10129), *Mycoplasma orale* (NCTC code 10112), *Spiroplasma citri* (NCTC code 10164), (Sartorius Stedim Biotech GmbH) were used. These lyophilized standards can be easily rehydrated by adding 1 ml of sample matrix for achieving a concentration of 10 CFU/ml. These preparations are intended for safe and reliable validation of Mycoplasma PCR assays. In these comparative studies DMEM with 5 % FCS was used as sample matrix.

For all three assays the DNA isolation process and the subsequent PCR set-up was done according to the manual of the manufacturer. The Microsart® AMP Extraction kit is based on a convenient silica column based protocol which can be completed within 30 minutes. The DNA isolation procedures of the competing products are based on DNA precipitation on a Magnetic Bead based protocol. Two of the three evaluated PCR assays include highly specific TaqMan probes; one of them is the Microsart® AMP Mycoplasma. The third assay includes SYBR Green in the detection mix which makes a melting curve analysis necessary after the DNA amplification cycles to differentiate between unspecific amplified DNA and Mycoplasma amplicon DNA.

Each species, *M. arginini, M. orale* and *S. citri*, was tested four times with each kit, which included the whole process of DNA isolation, Polymerase chain reaction and data analysis. The results are listed in Table 1.

Sample prep and PCR kit	Sample Material	Results	Standard deviations of Ct values	
Supplier T Kit for sample preparation Real-time PCR kit	10CFU *M. orale* Standard (Sartorius Stedim Biotech GmbH)	4/4 positive	0.8	0.5
	10CFU *S. citri* Standard (Sartorius Stedim Biotech GmbH)	4/4 positive	0.5	
	10CFU *M. arginini* Standard (Sartorius Stedim Biotech GmbH)	4/4 positive	0.3	
Supplier R Kit for sample preparation Real-time PCR kit	10CFU *M. orale* Standard (Sartorius Stedim Biotech GmbH)	4/4 positive	0.7	0.6
	10CFU *S. citri* Standard (Sartorius Stedim Biotech GmbH)	4/4 positive	0.6	
	10CFU *M. arginini* Standard (Sartorius Stedim Biotech GmbH)	4/4 positive	0.4	
Sartorius Stedim Biotech Microsart® AMP Extraction Microsart® AMP Mycoplasma	10CFU *M. orale* Standard (Sartorius Stedim Biotech GmbH)	4/4 positive	0.4	0.3
	10CFU *S. citri* Standard (Sartorius Stedim Biotech GmbH)	4/4 positive	0.3	
	10CFU *M. arginini* Standard (Sartorius Stedim Biotech GmbH)	4/4 positive	0.1	

Table 1: PCR results of DMEM+5% FCS with 10 CFU/ml of *M. arginini* / *M. orale* / *S. citri* using kits of three different suppliers

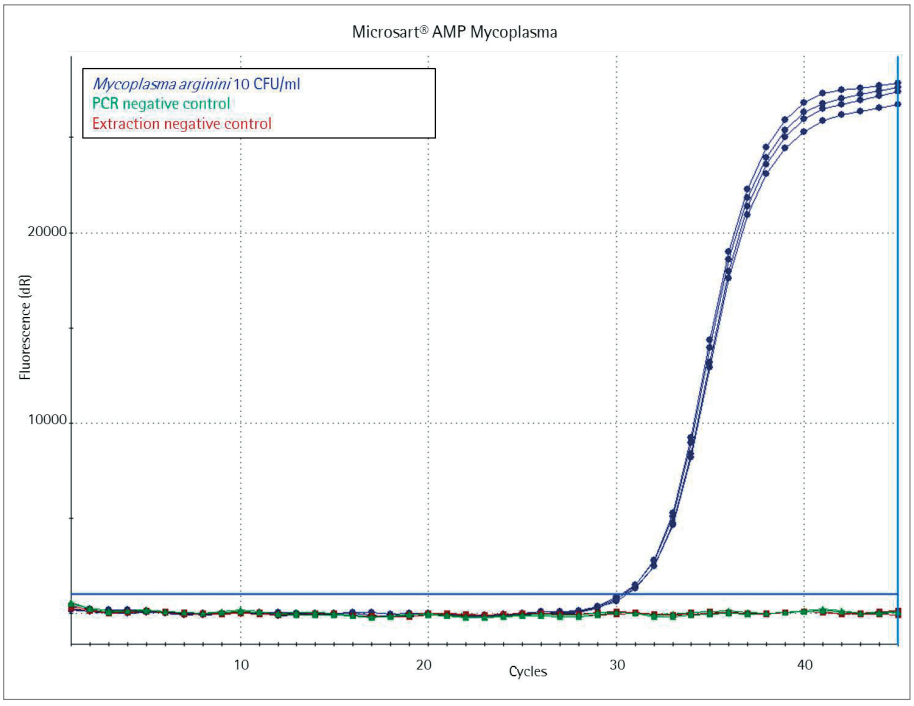

Figure 1: Amplification curves with the kit Microsart® AMP Mycoplasma, using the example of *Mycoplasma arginini* (10 CFU/ml in DMEM + 5 % FCS), PCR Cycler MxPro 3005P

Conclusion

In these studies Sartorius Microsart® AMP Extraction und Microsart® AMP Mycoplasma were benchmarked with Mycoplasma Real-time PCR kits incl. sample preparation of two other suppliers.

Each of the 36 DNA extracts showed positive signals in the subsequent PCR analysis. So all tested products are able to detect Mycoplasma contaminations with a sensitivity of at least 10 CFU per ml.

If having a closer look to the standard deviation within the results of one supplier, the Microsart® AMP Extraction followed by amplification with the Microsart® AMP Mycoplasma kit has led to the least variability and highest reproducibility. The PCR kits, but predominately the three different sample preparation methods might have contributed to the difference in reproducibility. Silica-membrane based procedures are known to be very robust and reliable even when processing highly complex sample matrices. DNA precipitation or Magnetic bead based protocols tend to be more laborious and less robust.

Another fact which contributes also to the reliability of the Microsart® Mycoplasma product range is that all PCR reagents can be stored at 4 to 8 °C. So, a short interruption in the cool chain would not lead to undesired thawing of the reagents which can have negative effects on the sensitivity of the PCR assay. In contrast the Mycoplasma detection kits of supplier T and supplier R have to be stored frozen at – 20°C.
When planning tests with these products additional waiting time for thawing of the PCR reagents have to be kept in mind. Furthermore it has to be considered if a kit which has to be stored at – 20°C was not used up completely within one PCR run, that several thawing and freezing cycles will also have negative effects on the PCR results.

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Correlation between colony forming units and genome copies of 9 different Mycoplasma species using quantified CFU and GC standards for validation

Application Note

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turning science into solutions

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Abstract

In this study the correlation between genome copies (GC) and colony forming units (CFU) of 9 different mycoplasma species has been investigated using Sartorius´ quantified CFU and GC standards. As PCR technology only detects genome copies (GC), a correlation between colony forming units (CFU) and GC is required by different authorities, i.e. Korean Food and Drug Administration (KFDA) or Pharmaceuticals and Medical Devices Agency PMDA, Japan, before an assay is accepted to be used in quality control of cell culture products. The results of this study show that the number of genome copies vary between Mycoplasma species, but have successfully been correlated to 20 CFU and 40 CFU respectively.

Introduction

Mycoplasma are the smallest free-living organisms. They belong to the bacterial class Mollicutes that are distinguished by their lack of cell wall. For that reason they are unaffected by many commonly antimicrobial agents such as beta-lactam antibiotics [1]. Mycoplasma are widespread contaminants in cell culture. In fact, depending on the laboratory, 10 % to 85 % of cell lines may be contaminated [2]. Due to their extremely basic genomes, mycoplasma live as parasites. They compete with host cells for biosynthetic precursors and nutrients and can alter DNA, RNA and protein synthesis and induce chromosomal alterations [2]. Given their tiny size (~0.3 – 0.8 µm) mycoplasma contamination cannot be visualized with a light microscope [1]. Moreover altered growth rates and morphological changes in infected cell cultures can be minimal or unapparent. Furthermore mycoplasma contaminated products represent a human health risk [2]. All these facts show clearly the high demand of routine mycoplasma detection.

Microsart® ATMP Mycoplasma enables a reliable and sensitive detection of mycoplasma DNA in cell cultures and cell culture derived biologicals, like autologous transplants, according to European Pharmacopeia 2.6.7. Regulations require comparability studies with compendial growth based methods. As PCR technology only detects genome copies (GC), a correlation between colony forming units (CFU) and GC is required by different authorities, i.e. Korean Food and Drug Administration (KFDA) or Pharmaceuticals and Medical Devices Agency PMDA, Japan.

In this study the correlation between CFU and GC of 9 different mycoplasma species was investigated using Sartorius´ quantified CFU and GC standards to facilitate implementation and approval of qPCR-based mycoplasma detection methods.

Materials and Methods

DNA Extraction of Microsart® Validation Standard

Each package of Microsart® Validation Standard contains 3 vials, each containing 10 CFU of the chosen Mycoplasma species. 250 µl Dulbecco's Modified Eagle Medium (DMEM) + 10 % fetal bovine serum (FBS) were added to two vials to prepare a suspension with a concentration of 40 CFU/ml. 500 µl DMEM +10 % FBS were added to one vial to prepare a suspension with a concentration of 20 CFU/ml. The DNA of the cell suspensions was extracted with Microsart® AMP Extraction kit in duplicates according to the protocol. The eluate was used directly for Microsart® ATMP Mycoplasma qPCR.

Standard curve with Microsart® Calibration Reagent

To quantify the DNA extracts of Microsart® Validation Standards it is necessary to generate a standard curve with known concentrations of genome copies (GC). Therefore Microsart® Calibration Reagents were used. The calibration reagents contain 10^6 GC/µl of the specific organism after rehydration. Dilution series have been prepared in Tris-buffer to achieve final concentrations of 5 GC/10 µl to 500 GC/10 µl.

Table 1: Product overview of Microsart® Validation Standards and Microsart® Calibration Reagents for different mycoplasma species. The Validation Standard contains 10 CFU per vial, the Calibration Reagent contains 106 GC/µl after rehydration.

			Catalog No.	Catalog No.
Mycoplasma species	**NCTC code**	**ATCC code**	**Microsart® Validation Standard**	**Microsart® Calibration Reagent**
Mycoplasma arginini	10129	23838	SMB95-2011	SMB95-2021
Mycoplasma orale	10112	23714	SMB95-2012	SMB95-2022
Mycoplasma gallisepticum	10115	19610	SMB95-2013	SMB95-2023
Mycoplasma pneumoniae	10119	15531	SMB95-2014	SMB95-2024
Mycoplasma synoviae	10124	25204	SMB95-2015	SMB95-2025
Mycoplasma fermentans	10117	19989	SMB95-2016	SMB95-2026
Mycoplasma hyorhinis	10130	17981	SMB95-2017	SMB95-2027
Acholeplasma laidlawii	10116	23206	SMB95-2018	SMB95-2028
Spiroplasma citri	10164	27556	SMB95-2019	SMB95-2029

Microsart® ATMP Mycoplasma qPCR

All lyophilized components were rehydrated. For one reaction 15 µl of Mycoplasma Mix were mixed with 1 µl Internal Control DNA. 15 µl of this mix were added to each PCR tube. Each test was carried out with at least two Non Template Controls (NTC) and samples in duplicate. Therefore 10 µl of sample or NTC were added to the PCR tubes with Master Mix respectively.

Microsart® ATMP Mycoplasma qPCR was performed on the CFX96 Touch Cycler (Bio-Rad; 45 cycles, 3 min 95 °C, 30 s 95 °, 30 s 55 °C, 45 s 60 °C). The mycoplasma DNA is indicated by an increasing fluorescence signal in the FAM™ channel. Internal Control DNA is detected in the ROX™ channel in the same tube to indicate a successful reaction in every individual PCR tube. The Analysis of the reaction was done with the CFX Manager Software (Bio-Rad). The limit of detection of all mycoplasma species listed in the EP/USP is ≤ 10 CFU/ml.

Results and Discussion

Figure 2 and 3 show exemplary amplification plots of *A. laidlawii*. On basis of the ct values (FAM™ channel) and concentrations of the standards the CFX Manager software created a standard curve (Fig. 1) with a linear equation. A regression coefficient of 0.983 is an indication for a good standard curve. The efficiency of an optimal qPCR is 100 %. In that case the amplicon DNA will be doubled in each cycle. According to the analysis of the CFX Software the exemplary qPCR run of *A. laidlawii* ran highly efficient with an efficiency of 101 % (see efficiency in Fig 1).

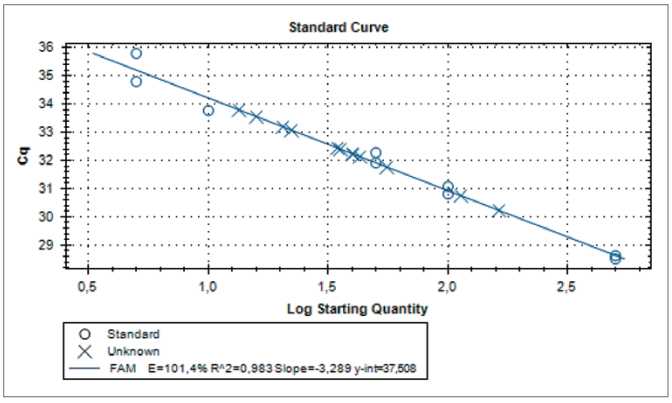

Figure 1: Exemplary standard curve of *Acholeplasma laidlawii* (Microsart® Calibration Reagent), using final Genomic Copy (GC) concentrations of 5 GC/10 µl to 500 GC/10 µl. generated with Microsart® ATMP Mycoplasma.

Each sample and Non-Template Control (NTC) showed an amplification of the internal control DNA and consequently fluorescence signal in the ROX™ channel (Ct<40; data not shown). A successfully PCR without inhibition was indicated. The NTC did not show a fluorescence signal in the FAM™ channel, as expected (see Fig. 2 and 3). Consequently a mycoplasma free preparation of the PCR reactions without cross-contamination was indicated.

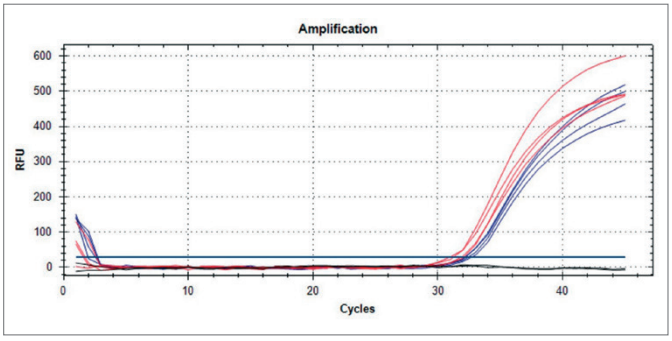

Figure 2: Exemplary amplification plot of *Acholeplasma laidlawii*, generated with Microsart® ATMP Mycoplasma qPCR. Fluorescence signals in FAM™ channel. Black Lines: Non template Control (NTC). Blue Lines: 20 CFU/ml of *A. laidlawii*. Red Lines: 40 CFU/ml of *A. laidlawii*.

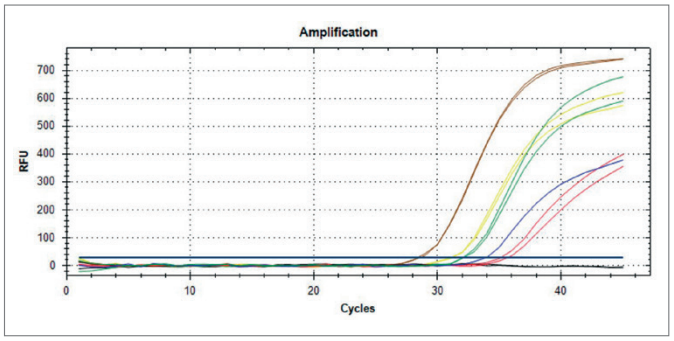

Figure 3: Exemplary amplification plot of *Acholeplasma laidlawii*, generated with Microsart® ATMP Mycoplasma qPCR. Fluorescence signals in FAM™ channel. Black Lines: Non template Control (NTC). Red: 5 GC/PCR. Blue: 10 GC/PCR. Green: 50 GC/PCR. Yellow: 100 GC/PCR. Brown: 500 GC/PCR.

20 CFU/ml and 40 CFU/ml of each mycoplasma species have been detected successfully in all samples (Assay LOD is ≤ 10 CFU/ml; determined during kit validation).

Based on the linear equation of the standard curve the software calculated the GC concentrations of the mycoplasma samples (20 CFU/ml and 40 CFU/ml extracts). In table 2 the average GC to CFU ratios of 9 different mycoplasma species are shown.

Table 2: Average GC to CFU ratio of 9 different mycoplasma species

Mycoplasma Species	**Average GC to CFU ratio**
Mycoplasma arginini	1.1 × 10 GC/CFU
Mycoplasma orale	3.5 × 10 GC/CFU
Mycoplasma gallisepticum	1.7 × 10 GC/CFU
Mycoplasma pneumoniae	4.3 × 10 GC/CFU
Mycoplasma synoviae	0.9 × 10 GC/CFU
Mycoplasma fermentans	1.2 × 10 GC/CFU
Mycoplasma hyorhinis	0.9 × 10 GC/CFU
Acholeplasma laidlawii	5.6 × 10 GC/CFU
Spiroplasma citri	6.8 × 10 GC/CFU

The study indicated that the GC/CFU ratio varied from species to species and lies within a range of 9 GC/CFU to 68 GC/CFU after DNA extraction.

Discussion

In this study the correlation between Genomic Copies (GC) and Colony Forming Units (CFU) of 9 different mycoplasma species has been investigated using Sartorius quantified GC and CFU standards. A correlation between CFU and GC is required by different authorities, i.e. Korean Food and Drug Administration (KFDA) or Pharmaceuticals and Medical Devices Agency PMDA, Japan, for assay approval. These results demonstrate a good data basis. Nevertheless it should be kept in mind that significant variations within the GC:CFU ratio might be observed if other media | matrices are used for the CFU spikes which affects the DNA isolation efficiency or other conditions are used for correlation. The results of this study indicate a higher GC than CFU number, as expected. The theoretical GC:CFU ratio should be 1:1, as one GC per cell should ideally be detected. Practically, this ratio is not realizable even if mycoplasma cultures are harvested during early logarithmic growth to prevent detection of DNA from dead cells in the preparation. This non-equal ratio arises because a significant number of the mycoplasma cells would not grow to a colony in culture and remain undetected (i.e. stressed or viable but non-culturable cells). Furthermore mycoplasma cells tend to form agglomerates, which would be detected as 1 CFU, but in fact combine several cells and therefore several GC. Both scenarios lead to a significant underestimation of the realistic Mycoplasma cell number in the sampled cell culture, as only a portion of the cells would grow to form a CFU. Non-culturable species or viable but non-culturable cells could lead to false-negative results using a growth-based method. Undetected Mycoplasma contamination because of false-negative results in growth based methods can result in unsafe products with potential infection risks especially for patients with immunodeficiency. This study shows that the correlation between GC and CFU can successfully be demonstrated and easily be implemented during validation. Furthermore detection of GC by PCR shows a more realistic result of the real contamination level in the respective sample and therefore directly contributes to drug safety.

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Benchmarking of the Microsart® AMP Mycoplasma kit with products of two other suppliers

Application Note

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turning science into solutions

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Introduction

Among the world's smallest bacteria Mycoplasmas are capable of independent reproduction. They belong to the class of Mollicutes and have a very slow and parasitic growth. The contamination of cell cultures remains a major problem. An array of physiological and biochemical parameters are affected by the presence of Mycoplasma in cells cultures. The infection causes changes in metabolism, growth, viability, macromolecule synthesis, morphology etc. and therefore a sensitive routine testing for contamination of cell cultures is essential.

The traditional growth-based method requires a cultivation time of at least 28 days before a contamination can be ruled out with certainty. In comparison, Nucleic Acid Amplification Techniques (NAT) allow to reduce time to results to just hours. As an alternative to the culture method the NAT test system must be shown to detect 10 CFU/mL of Mycoplasma. For this reason the capability of three different Mycoplasma PCR kits to detect spikes of not higher than 10 CFU per ml in Dulbecco's Modified Eagle Medium was tested in this study.

Experimental Set-up and Results

During this study the Microsart® AMP Mycoplasma kit inclusive sample preparation with the kit Microsart® AMP Extraction (Sartorius Stedim Biotech GmbH) were benchmarked with two other Mycoplasma detection kits which are also based on DNA isolation with subsequent Real-time PCR analysis. As test material the 10CFU Sensitivity Standards *Mycoplasma arginini* (NCTC code 10129), *Mycoplasma orale* (NCTC code 10112), *Spiroplasma citri* (NCTC code 10164), (Sartorius Stedim Biotech GmbH) were used. These lyophilized standards can be easily rehydrated by adding 1 ml of sample matrix for achieving a concentration of 10 CFU/ml. These preparations are intended for safe and reliable validation of Mycoplasma PCR assays. In these comparative studies DMEM with 5 % FCS was used as sample matrix.

For all three assays the DNA isolation process and the subsequent PCR set-up was done according to the manual of the manufacturer. The Microsart® AMP Extraction kit is based on a convenient silica column based protocol which can be completed within 30 minutes. The DNA isolation procedures of the competing products are based on DNA precipitation on a Magnetic Bead based protocol. Two of the three evaluated PCR assays include highly specific TaqMan probes; one of them is the Microsart® AMP Mycoplasma. The third assay includes SYBR Green in the detection mix which makes a melting curve analysis necessary after the DNA amplification cycles to differentiate between unspecific amplified DNA and Mycoplasma amplicon DNA.

Each species, *M. arginini, M. orale* and *S. citri*, was tested four times with each kit, which included the whole process of DNA isolation, Polymerase chain reaction and data analysis. The results are listed in Table 1.

Sample prep and PCR kit	Sample Material	Results	Standard deviations of Ct values	
Supplier T Kit for sample preparation Real-time PCR kit	10CFU *M. orale* Standard (Sartorius Stedim Biotech GmbH)	4/4 positive	0.8	0.5
	10CFU *S. citri* Standard (Sartorius Stedim Biotech GmbH)	4/4 positive	0.5	
	10CFU *M. arginini* Standard (Sartorius Stedim Biotech GmbH)	4/4 positive	0.3	
Supplier R Kit for sample preparation Real-time PCR kit	10CFU *M. orale* Standard (Sartorius Stedim Biotech GmbH)	4/4 positive	0.7	0.6
	10CFU *S. citri* Standard (Sartorius Stedim Biotech GmbH)	4/4 positive	0.6	
	10CFU *M. arginini* Standard (Sartorius Stedim Biotech GmbH)	4/4 positive	0.4	
Sartorius Stedim Biotech Microsart® AMP Extraction Microsart® AMP Mycoplasma	10CFU *M. orale* Standard (Sartorius Stedim Biotech GmbH)	4/4 positive	0.4	0.3
	10CFU *S. citri* Standard (Sartorius Stedim Biotech GmbH)	4/4 positive	0.3	
	10CFU *M. arginini* Standard (Sartorius Stedim Biotech GmbH)	4/4 positive	0.1	

Table 1: PCR results of DMEM+5% FCS with 10 CFU/ml of *M. arginini* / *M. orale* / *S. citri* using kits of three different suppliers

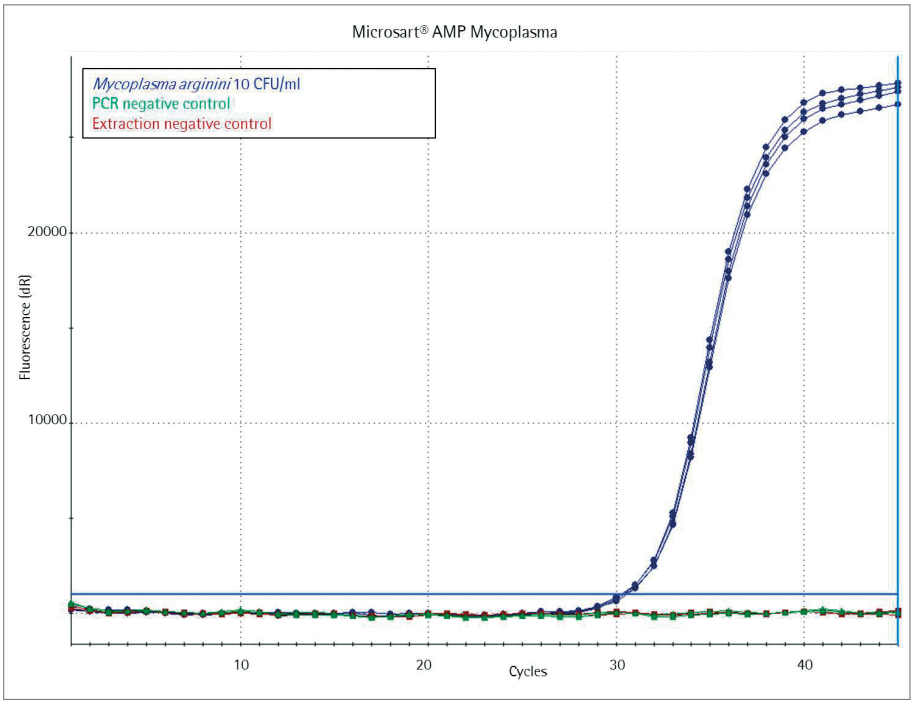

Figure 1: Amplification curves with the kit Microsart® AMP Mycoplasma, using the example of *Mycoplasma arginini* (10 CFU/ml in DMEM + 5 % FCS), PCR Cycler MxPro 3005P

Conclusion

In these studies Sartorius Microsart® AMP Extraction und Microsart® AMP Mycoplasma were benchmarked with Mycoplasma Real-time PCR kits incl. sample preparation of two other suppliers.

Each of the 36 DNA extracts showed positive signals in the subsequent PCR analysis. So all tested products are able to detect Mycoplasma contaminations with a sensitivity of at least 10 CFU per ml.

If having a closer look to the standard deviation within the results of one supplier, the Microsart® AMP Extraction followed by amplification with the Microsart® AMP Mycoplasma kit has led to the least variability and highest reproducibility. The PCR kits, but predominately the three different sample preparation methods might have contributed to the difference in reproducibility. Silica-membrane based procedures are known to be very robust and reliable even when processing highly complex sample matrices. DNA precipitation or Magnetic bead based protocols tend to be more laborious and less robust.

Another fact which contributes also to the reliability of the Microsart® Mycoplasma product range is that all PCR reagents can be stored at 4 to 8 °C. So, a short interruption in the cool chain would not lead to undesired thawing of the reagents which can have negative effects on the sensitivity of the PCR assay. In contrast the Mycoplasma detection kits of supplier T and supplier R have to be stored frozen at – 20°C.
When planning tests with these products additional waiting time for thawing of the PCR reagents have to be kept in mind. Furthermore it has to be considered if a kit which has to be stored at – 20°C was not used up completely within one PCR run, that several thawing and freezing cycles will also have negative effects on the PCR results.

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Correlation between colony forming units and genome copies of 9 different Mycoplasma species using quantified CFU and GC standards for validation

Application Note

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turning science into solutions

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June 2017

Abstract

In this study the correlation between genome copies (GC) and colony forming units (CFU) of 9 different mycoplasma species has been investigated using Sartorius´ quantified CFU and GC standards. As PCR technology only detects genome copies (GC), a correlation between colony forming units (CFU) and GC is required by different authorities, i.e. Korean Food and Drug Administration (KFDA) or Pharmaceuticals and Medical Devices Agency PMDA, Japan, before an assay is accepted to be used in quality control of cell culture products. The results of this study show that the number of genome copies vary between Mycoplasma species, but have successfully been correlated to 20 CFU and 40 CFU respectively.

Introduction

Mycoplasma are the smallest free-living organisms. They belong to the bacterial class Mollicutes that are distinguished by their lack of cell wall. For that reason they are unaffected by many commonly antimicrobial agents such as beta-lactam antibiotics [1]. Mycoplasma are widespread contaminants in cell culture. In fact, depending on the laboratory, 10 % to 85 % of cell lines may be contaminated [2]. Due to their extremely basic genomes, mycoplasma live as parasites. They compete with host cells for biosynthetic precursors and nutrients and can alter DNA, RNA and protein synthesis and induce chromosomal alterations [2]. Given their tiny size (~0.3 – 0.8 µm) mycoplasma contamination cannot be visualized with a light microscope [1]. Moreover altered growth rates and morphological changes in infected cell cultures can be minimal or unapparent. Furthermore mycoplasma contaminated products represent a human health risk [2]. All these facts show clearly the high demand of routine mycoplasma detection.

Microsart® ATMP Mycoplasma enables a reliable and sensitive detection of mycoplasma DNA in cell cultures and cell culture derived biologicals, like autologous transplants, according to European Pharmacopeia 2.6.7. Regulations require comparability studies with compendial growth based methods. As PCR technology only detects genome copies (GC), a correlation between colony forming units (CFU) and GC is required by different authorities, i.e. Korean Food and Drug Administration (KFDA) or Pharmaceuticals and Medical Devices Agency PMDA, Japan.

In this study the correlation between CFU and GC of 9 different mycoplasma species was investigated using Sartorius´ quantified CFU and GC standards to facilitate implementation and approval of qPCR-based mycoplasma detection methods.

Materials and Methods

DNA Extraction of Microsart® Validation Standard

Each package of Microsart® Validation Standard contains 3 vials, each containing 10 CFU of the chosen Mycoplasma species. 250 µl Dulbecco's Modified Eagle Medium (DMEM) + 10 % fetal bovine serum (FBS) were added to two vials to prepare a suspension with a concentration of 40 CFU/ml. 500 µl DMEM +10 % FBS were added to one vial to prepare a suspension with a concentration of 20 CFU/ml. The DNA of the cell suspensions was extracted with Microsart® AMP Extraction kit in duplicates according to the protocol. The eluate was used directly for Microsart® ATMP Mycoplasma qPCR.

Standard curve with Microsart® Calibration Reagent

To quantify the DNA extracts of Microsart® Validation Standards it is necessary to generate a standard curve with known concentrations of genome copies (GC). Therefore Microsart® Calibration Reagents were used. The calibration reagents contain 10^6 GC/µl of the specific organism after rehydration. Dilution series have been prepared in Tris-buffer to achieve final concentrations of 5 GC/10 µl to 500 GC/10 µl.

Table 1: Product overview of Microsart® Validation Standards and Microsart® Calibration Reagents for different mycoplasma species. The Validation Standard contains 10 CFU per vial, the Calibration Reagent contains 106 GC/µl after rehydration.

			Catalog No.	Catalog No.
Mycoplasma species	**NCTC code**	**ATCC code**	**Microsart® Validation Standard**	**Microsart® Calibration Reagent**
Mycoplasma arginini	10129	23838	SMB95-2011	SMB95-2021
Mycoplasma orale	10112	23714	SMB95-2012	SMB95-2022
Mycoplasma gallisepticum	10115	19610	SMB95-2013	SMB95-2023
Mycoplasma pneumoniae	10119	15531	SMB95-2014	SMB95-2024
Mycoplasma synoviae	10124	25204	SMB95-2015	SMB95-2025
Mycoplasma fermentans	10117	19989	SMB95-2016	SMB95-2026
Mycoplasma hyorhinis	10130	17981	SMB95-2017	SMB95-2027
Acholeplasma laidlawii	10116	23206	SMB95-2018	SMB95-2028
Spiroplasma citri	10164	27556	SMB95-2019	SMB95-2029

Microsart® ATMP Mycoplasma qPCR

All lyophilized components were rehydrated. For one reaction 15 µl of Mycoplasma Mix were mixed with 1 µl Internal Control DNA. 15 µl of this mix were added to each PCR tube. Each test was carried out with at least two Non Template Controls (NTC) and samples in duplicate. Therefore 10 µl of sample or NTC were added to the PCR tubes with Master Mix respectively.

Microsart® ATMP Mycoplasma qPCR was performed on the CFX96 Touch Cycler (Bio-Rad; 45 cycles, 3 min 95 °C, 30 s 95 °, 30 s 55 °C, 45 s 60 °C). The mycoplasma DNA is indicated by an increasing fluorescence signal in the FAM™ channel. Internal Control DNA is detected in the ROX™ channel in the same tube to indicate a successful reaction in every individual PCR tube. The Analysis of the reaction was done with the CFX Manager Software (Bio-Rad). The limit of detection of all mycoplasma species listed in the EP/USP is ≤ 10 CFU/ml.

Results and Discussion

Figure 2 and 3 show exemplary amplification plots of *A. laidlawii*. On basis of the ct values (FAM™ channel) and concentrations of the standards the CFX Manager software created a standard curve (Fig. 1) with a linear equation. A regression coefficient of 0.983 is an indication for a good standard curve. The efficiency of an optimal qPCR is 100 %. In that case the amplicon DNA will be doubled in each cycle. According to the analysis of the CFX Software the exemplary qPCR run of *A. laidlawii* ran highly efficient with an efficiency of 101 % (see efficiency in Fig 1).

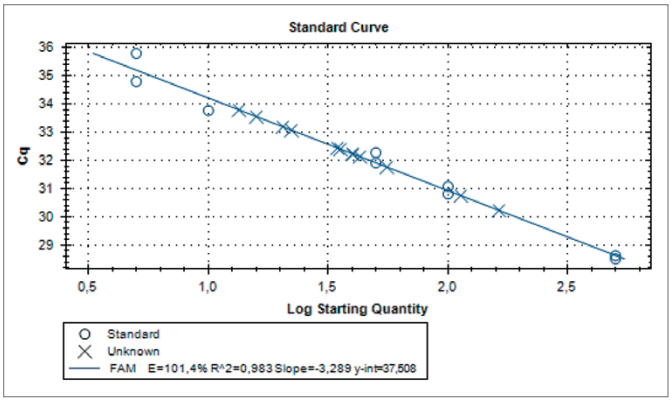

Figure 1: Exemplary standard curve of *Acholeplasma laidlawii* (Microsart® Calibration Reagent), using final Genomic Copy (GC) concentrations of 5 GC/10 µl to 500 GC/10 µl. generated with Microsart® ATMP Mycoplasma.

Each sample and Non-Template Control (NTC) showed an amplification of the internal control DNA and consequently fluorescence signal in the ROX™ channel (Ct<40; data not shown). A successfully PCR without inhibition was indicated. The NTC did not show a fluorescence signal in the FAM™ channel, as expected (see Fig. 2 and 3). Consequently a mycoplasma free preparation of the PCR reactions without cross-contamination was indicated.

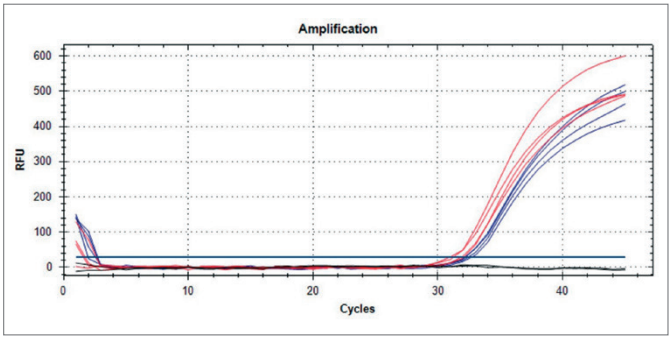

Figure 2: Exemplary amplification plot of *Acholeplasma laidlawii*, generated with Microsart® ATMP Mycoplasma qPCR. Fluorescence signals in FAM™ channel. Black Lines: Non template Control (NTC). Blue Lines: 20 CFU/ml of *A. laidlawii*. Red Lines: 40 CFU/ml of *A. laidlawii*.

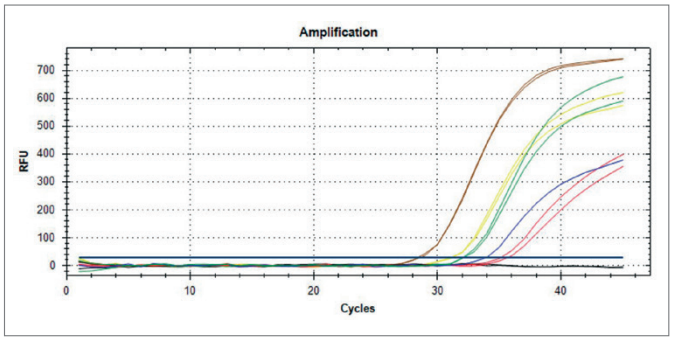

Figure 3: Exemplary amplification plot of *Acholeplasma laidlawii*, generated with Microsart® ATMP Mycoplasma qPCR. Fluorescence signals in FAM™ channel. Black Lines: Non template Control (NTC). Red: 5 GC/PCR. Blue: 10 GC/PCR. Green: 50 GC/PCR. Yellow: 100 GC/PCR. Brown: 500 GC/PCR.

20 CFU/ml and 40 CFU/ml of each mycoplasma species have been detected successfully in all samples (Assay LOD is ≤ 10 CFU/ml; determined during kit validation).

Based on the linear equation of the standard curve the software calculated the GC concentrations of the mycoplasma samples (20 CFU/ml and 40 CFU/ml extracts). In table 2 the average GC to CFU ratios of 9 different mycoplasma species are shown.

Table 2: Average GC to CFU ratio of 9 different mycoplasma species

Mycoplasma Species	**Average GC to CFU ratio**
Mycoplasma arginini	1.1 × 10 GC/CFU
Mycoplasma orale	3.5 × 10 GC/CFU
Mycoplasma gallisepticum	1.7 × 10 GC/CFU
Mycoplasma pneumoniae	4.3 × 10 GC/CFU
Mycoplasma synoviae	0.9 × 10 GC/CFU
Mycoplasma fermentans	1.2 × 10 GC/CFU
Mycoplasma hyorhinis	0.9 × 10 GC/CFU
Acholeplasma laidlawii	5.6 × 10 GC/CFU
Spiroplasma citri	6.8 × 10 GC/CFU

The study indicated that the GC/CFU ratio varied from species to species and lies within a range of 9 GC/CFU to 68 GC/CFU after DNA extraction.

Discussion

In this study the correlation between Genomic Copies (GC) and Colony Forming Units (CFU) of 9 different mycoplasma species has been investigated using Sartorius quantified GC and CFU standards. A correlation between CFU and GC is required by different authorities, i.e. Korean Food and Drug Administration (KFDA) or Pharmaceuticals and Medical Devices Agency PMDA, Japan, for assay approval. These results demonstrate a good data basis. Nevertheless it should be kept in mind that significant variations within the GC:CFU ratio might be observed if other media | matrices are used for the CFU spikes which affects the DNA isolation efficiency or other conditions are used for correlation. The results of this study indicate a higher GC than CFU number, as expected. The theoretical GC:CFU ratio should be 1:1, as one GC per cell should ideally be detected. Practically, this ratio is not realizable even if mycoplasma cultures are harvested during early logarithmic growth to prevent detection of DNA from dead cells in the preparation. This non-equal ratio arises because a significant number of the mycoplasma cells would not grow to a colony in culture and remain undetected (i.e. stressed or viable but non-culturable cells). Furthermore mycoplasma cells tend to form agglomerates, which would be detected as 1 CFU, but in fact combine several cells and therefore several GC. Both scenarios lead to a significant underestimation of the realistic Mycoplasma cell number in the sampled cell culture, as only a portion of the cells would grow to form a CFU. Non-culturable species or viable but non-culturable cells could lead to false-negative results using a growth-based method. Undetected Mycoplasma contamination because of false-negative results in growth based methods can result in unsafe products with potential infection risks especially for patients with immunodeficiency. This study shows that the correlation between GC and CFU can successfully be demonstrated and easily be implemented during validation. Furthermore detection of GC by PCR shows a more realistic result of the real contamination level in the respective sample and therefore directly contributes to drug safety.

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Mycoplasma Assays for qPCR

The new Gold Standard

Mycoplasma qPCR assays offer gold standard performance, design and configuration flexibility, affordability, and manufacturing quality.

Flexibility

Select the product you need and take advantage of the protocol which fits perfectly to your sample simply by design.

- Fast and reliable screening of cell cultures in research, ATMPs or high end confidence testing in production – well designed kits for any need.
- Protocols for testing 2 µl, 200 µl or up to 18 ml are available.
- Open system, no hardware bundle – take advantage of the qPCR you already own and keep your investment low.

Convenience

All three kits are based on easy-to-use real-time PCR technology for fast and reliable results.

- The TaqMan® assay design reduces the number of reactions needed, as it enables the use of internal controls. This increases the reliability and reduces the costs of the tests.
- The lyophilized Mycoplasma Mix contains all reaction components including the polymerase and can be easily reconstituted with the rehydration buffer included in the kit. It comes in aliquots of 25 reactions for highest convenience and long term stability.

Performance

By teaming up with Minerva Biolabs, Sartorius has gained a partner with long standing experience and an extensive research background in qPCR – providing performance-guaranteed reagents and results.

- Comprehensive validation according to European Pharmacopoeia 2.6.7.
- Challenged in proficiency tests and in-depth robustness studies.

Kit Selection

Features	Microsart® RESEARCH Mycoplasma	Microsart® ATMP Mycoplasma	Microsart® AMP Mycoplasma
Recommended use | scope	Testing of cell culture materials in research and development	Testing of autologous cell transplants (ATMPs)	Regulated in-process and lot-release testing
Type of PCR	5′ nuclease assay for qPCR	5′ nuclease assay for qPCR	5′ nuclease assay for qPCR
Device requirements	None. Kit can be applied on any qPCR cycler suitable to detect FAM™ and ROX™ dyes	None. Kit can be applied on any qPCR cycler suitable to detect FAM™ and ROX™ dyes	Kit can be applied on any qPCR cycler suitable to detect FAM™ and ROX™ dyes and accepting 100 µl reaction volume
Kit components	– lyophilized primers | nucleotides | probes | polymerase | internal amplification control, aliquoted in 25 reactions – rehydration buffer – lyophilized positive control – PCR grade water	– lyophilized primers | nucleotides | probes | polymerase, aliquoted in 25 reactions – internal amplification control usable as DNA extraction control – rehydration buffer – lyophilized positive control – PCR grade water	– lyophilized primers | nucleotides | probes | polymerase, aliquoted in 25 reactions – internal amplification control usable as DNA extraction control, aliquoted in 25 reactions – rehydration buffer – lyophilized positive control, aliquoted in 25 reactions – PCR grade water
Package sizes	Cat.-No. SMB95-1005 25 tests Cat.-No. SMB95-1006 100 tests	Cat.-No. SMB95-1003 25 tests Cat.-No. SMB95-1004 100 tests	Cat.-No. SMB95-1001 25 tests Cat.-No. SMB95-1002 100 tests
Sample volume	2 µl	200 µl	200 µl to 18 ml
Sample volume per PCR	2 µl	10 µl	50 µl
EP 2.6.7 compliance	No	Yes, for ATMPs only	Yes, comprehensive
Validation	Specificity and sensitivity based on genome units	Specificity and sensitivity based on CFU for all EP listed mycoplasma species, robustness for ATMPs only	Comprehensive specificity testing including production cell lines and various bacteria species; sensitivity based on CFU for all EP listed mycoplasma species; robustness for different types of cell culture materials and supplements
Result evaluation	Cycler based, real-time PCR; validated protocols available	Cycler based, real-time PCR; validated protocols available	Cycler based, real-time PCR; validated protocols available
Required consumables	PCR reaction tubes	PCR reaction tubes	PCR reaction tubes
Optional consumables	None	– 10CFU™ Sensitivity Standards for validation and performance control (e.g. SMB95-2011) – Microsart® AMP Extraction kit (Cat. No. SMB95-2003)	– 10CFU™ Sensitivity Standards for validation and performance control (e.g. SMB95-2011) – Microsart® AMP Extraction kit (Cat. No. SMB95-2003) – Vivaspin® 6 or 20 (e.g. VS0641, VS2041) in combination with Microsart® AMP Coating Buffer (SMB95-2002) to process volumes higher than 200 µl
Shelf life and storage	Components can be stored at +2 to +8 °C for at least 12 months. After rehydration the reagents must be stored at -18 °C	Components can be stored at +2 to +8 °C for at least 12 months. After rehydration the reagents must be stored at -18 °C	Components can be stored at +2 to +8 °C for at least 12 months. After rehydration the reagents must be stored at -18 °C

Microsart® RESEARCH Mycoplasma, Microsart® ATMP Mycoplasma and Microsart® AMP Mycoplasma are distributed by Sartorius Lab Products and Services (www.sartorius.com). Please contact your local Sartorius representative for local availability and ordering. Contact Sartorius Stedim Biotech or Minerva Biolabs for technical requests or any assistance.

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Instructions for Use

Microsart® ATMP Bacteria

Bacteria Detection Kit for qPCR

Prod. No. SMB95-1008

Reagents for 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 100 tests
	Manufacturer

Contents

1. Intended Use

Microsart® ATMP Bacteria kit is designed for the direct detection of bacteria in cell cultures, cell culture derived biologicals and ATMPs (e.g. autologous transplants), based on real-time PCR (qPCR).

2. Explanation of the Test

Microsart® ATMP Bacteria utilizes qPCR as the method of choice for sensitive and robust detection of bacterial contamination. 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 2.5 hours (including DNA extraction: 3.5 hours). In contrast to the culture method, samples do not need to contain vital bacteria.

3. Test Principle

Bacteria are specifically detected by amplifying a highly conserved rRNA operon, or more specifically, a 16S rRNA coding region in the bacterial genome. The specific amplification is detected at 520 nm (FAM™ channel). The kit includes primer and FAM™ labeled probes which allow the specific detection of many bacterial species. The polymerase is part of the Bacteria Mix. 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. 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).

4. Notes on the Test Procedure

1. For *in vitro* use in research and quality control. This kit may be disposed of according to local regulations.

2. This kit should be used only by trained persons. You should wear a clean lab coat and use disposable gloves at all times while performing the assay.

3. To avoid DNA cross-contaminations, the complete test must be performed under sterile and DNA-free conditions (see chapter 4.1 for detailed information).

4. In case of working with living bacteria strains, the local regulatory requirements for S2 labs must be considered.

5. Attention: by aliquoting and freezing your samples you run a high risk of contamination. This should therefore be avoided if possible.

6. This leaflet must be widely understood for a successful use of Microsart® ATMP Bacteria. 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.

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

8. Inhibition may be caused by the sample matrix, but also by sample elution buffer of DNA extraction kits which are not recommended or validated. Please note that by using DNA extraction kits which are not validated you run a high risk of obtaining false-positive or false-negative results.

9. 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.

10. The use of control samples is advised to secure the day-to-day validity of results. The controls should be carried out in the same manner as the samples.

4.1 Handling and Equipment Recommendations

To avoid false positive results due to improper handling the following actions are recommended:

1. To avoid DNA cross contaminations the complete test must be performed under sterile and DNA-free conditions. Therefore, we recommend the use of an isolator/glovebox with an airlock.

2. The isolator/glovebox should be cleaned thoroughly with PCR Clean™ (Minerva Biolabs, Prod. No. 15-2025) or PCR Clean™ Wipes (Minerva Biolabs, Prod. No. 15-2001) before and during the working process.

3. All materials, which are introduced into the isolator/glovebox should be cleaned thoroughly with PCR Clean™. Don´t forget to clean the airlock with PCR Clean™. Pipettes and gloves should be cleaned thoroughly with PCR Clean™ Wipes prior and during the process.

4. Avoid working above open tubes and avoid air turbulences due to rapid movements.

5. Be careful when opening the tubes. Do not touch the inner surface of the lid.

5. Reagents

Each kit contains reagents for 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. Lyophilized components must be stored at ≤ -18 ° C after rehydration. Protect the Bacteria Mix from light.

Kit Component Label Information	100 Reactions Order No. SMB95-1008	Cap Color
Bacteria Mix	4 x lyophilized	red
Rehydration Buffer	4 x 0.5 ml	blue
Positive Control DNA	1 x lyophilized	green
Internal Control DNA	4 x lyophilized	yellow
PCR grade Water	5 x 1.5 ml	white

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

6. Needed but not Included

Microsart® ATMP Bacteria contains the reagents, including negative and positive controls and polymerase as a component of the Bacteria Mix, to perform the test. General industrial supplies and reagents, usually available in PCR laboratories are not included:

Consumables

- Laboratory gloves
- PCR Clean™ (Minerva Biolabs, Prod. No. 15-2025) and PCR Clean™ wipes (Minerva Biolabs, Prod. No. 15-2001)
- DNA-free PCR reaction tubes (PCR 8-SoftStrips with attached caps from Biozym are recommended: 0.1 ml Low Profile, Prod. No. 710975 and 0.2 ml High Profile, Prod. No. 710970)
- DNA-free pipette filter tips that must be free from bacterial DNA (Biosphere® filter tips from Sarstedt are recommended: 0.5-20 µl, Prod No. 70.1116.210; 2-100 µl, Prod No. 70.760.212; 20-300 µl, Prod. No. 70.765.210; 100-1000 µl. Prod. No. 70.762.211)
- Microsart® Bacteria Extraction kit, a DNA-free extraction kit, Sartorius Prod. No. SMB95-2001.

Equipment

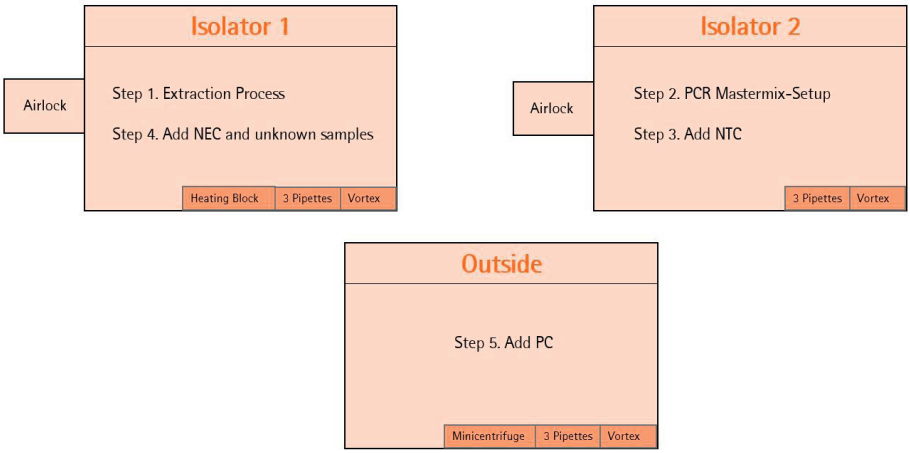
- Isolator/glovebox (further information, supplier and prices are available on request, please contact PCR@sartorius.com)
- qPCR device with filter sets for the detection of the fluorescence dyes FAM™ and ROX™ and suitable for 25 µl PCR reaction volumes
- Minicentrifuge for reaction tubes and PCR tubes
- Vortex mixer
- Rack for 1.5 ml tubes and for PCR-tube strips
- Pipettes (Sartorius)

mechanical	0.5 – 10 µl Sartorius Prod. No. LH-729020
	10 – 100 µl Sartorius Prod. No. LH-729050
	100 – 1000 µl Sartorius Prod. No. LH-729070
or electrical	0,2 – 10 µl Sartorius Prod. No. 735021
	10 – 300 µl Sartorius Prod. No. 735061
	50 – 1000 µl Sartorius Prod. No. 735081

If you use the Microsart® Bacteria Extraction kit you additionally need the following equipment:

- Isolator/glovebox (for extraction process)
- Vortex mixer
- Set of 3 pipettes (10 µl, 100 µl and 1000 µl; see page 10 for order information)
- Microcentrifuge for 1.5 ml reaction tubes (Centrisart A-14, Sartorius Prod. No. A-14-1EU)
- Heat Block
- Rack for 1.5 ml tubes

Schematical Overview of technical setup and experimental design



It is also possible to connect Isolator 1 and Isolator 2 via an airlock so that you can transfer your PCR-tubes after Step 3 directly from Isolator 2 into Isolator 1. Please note that you need an additional airlock for Isolator 2 in this case.

7. Specimen

Studies showed the strict requirement of DNA extraction for any kind of sample to achieve highest sensitivity. For most test materials a DNA extraction method is available providing templates suitable for PCR. However, most of the DNA extraction kits available on the market are not free of DNA contaminations. Therefore, we recommend the combination of Microsart® ATMP Bacteria with the Microsart® Bacteria Extraction kit (Prod. No. SMB95-2001), a unique state-of-the-art DNA extraction method, which eliminates the risk of DNA contaminations, facilitating the detection of bacteria in cell culture and ATMPs via qPCR.

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 is prepared. For this, Microsart® Calibration Reagents are recommended (see Chapter 12: Related Products for ordering information). All reagents and samples must be equilibrated at +2 to +8 °C prior use.

8.1 Recommendation for product release testing

The extraction process (Microsart® Bacteria Extraction) should be carried out with a negative extraction control (NEC) and samples in duplicates. Additionally the PCR test should include a PCR negative control (NTC) and a PCR positive control.

DNA extraction	PCR
2 x Sample	2 x Sample
1 x Negative Extraction Control	1 x Negative Extraction Control
	1 x PCR Positive Control
	1 x PCR Negative Control
∑ 3 extractions	∑ 5 PCR reactions

8.2 Rehydration of the Reagents

After reconstitution, reagents must be stored at ≤ -18 °C. In order to avoid repeated freezing and thawing, storage in appropriate aliquots is recommended.

1.	Bacteria Mix Internal Control DNA Positive Control DNA	red cap yellow cap green cap	Centrifuge briefly
2.	Bacteria Mix	red cap	Add 390 µl Rehydration Buffer (blue cap)
3.	Internal Control DNA	yellow cap	Add 800 µl PCR grade Water (white cap)
4.	Positive Control DNA	green cap	Add 300 µl PCR grade Water (white cap)
5.	Bacteria Mix Internal Control DNA Positive Control DNA	red cap yellow cap green cap	Incubate 5 min at room temperature
6.	Bacteria Mix Internal Control DNA Positive Control DNA	red cap yellow cap green cap	Vortex briefly

8.3 Preparation of the Reaction Mix

Preparation of the master mix and sample loading should not take more than 45 minutes to avoid a reduction in the fluorescent signal. The pipetting sequence should be respected and the tubes closed after each sample load.

The total volume per reaction is 25 µl including 10 µl sample. Calculations of reaction setup should include positive (PC) and negative controls (NTC).

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

1. Prepare the master mix at room temperature, by adding 26µl of Internal Control DNA (yellow cap) directly in the Bacteria Mix tube (red cap).
2. Homogenize the reaction mix by tapping carefully against the tube. Spin briefly.
3. Add 15 µl of the mastermix to 25 PCR tubes. Close PCR tubes. For storage, freeze any mastermix aliquots you do not need for the current assay.
   Attention: in order to avoid contaminations we recommend to freeze the PCR tubes containing the master mix in a closed and clean PCR rack.

Attention:
If the Internal Control DNA was added to the sample during DNA extraction, add 15 µl of the Bacteria Mix (red cap) directly to each PCR tube. Attention: Don´t forget to add 1µl of Internal Control DNA to NTC and PC.

8.4 Loading the Test Tubes

1. Negative controls: add 10 µl Suspension Buffer (violet cap) from the DNA extraction kit or PCR grade Water (white cap). Seal tube before proceeding with the samples.
   Attention: Negative controls should be processed in the isolator/glovebox used for mastermix setup.
   Attention: in order to avoid contaminations make sure to reserve one of the PCR grade water tube (white cap) exclusively to the negative controls.
2. Sample reaction: add 10 µl of sample. Seal tube tightly before proceeding.
   Attention: Samples, including NECs, should be added to the reaction in the isolator/glovebox used for DNA extraction.
3. Positive control: add 10 µl Positive Control DNA (green cap).
   Attention: Positive controls should not be handled in the isolator/glovebox used for mastermix setup or DNA extraction.
4. Close and spin all PCR tubes briefly, load the qPCR cycler and start the program.

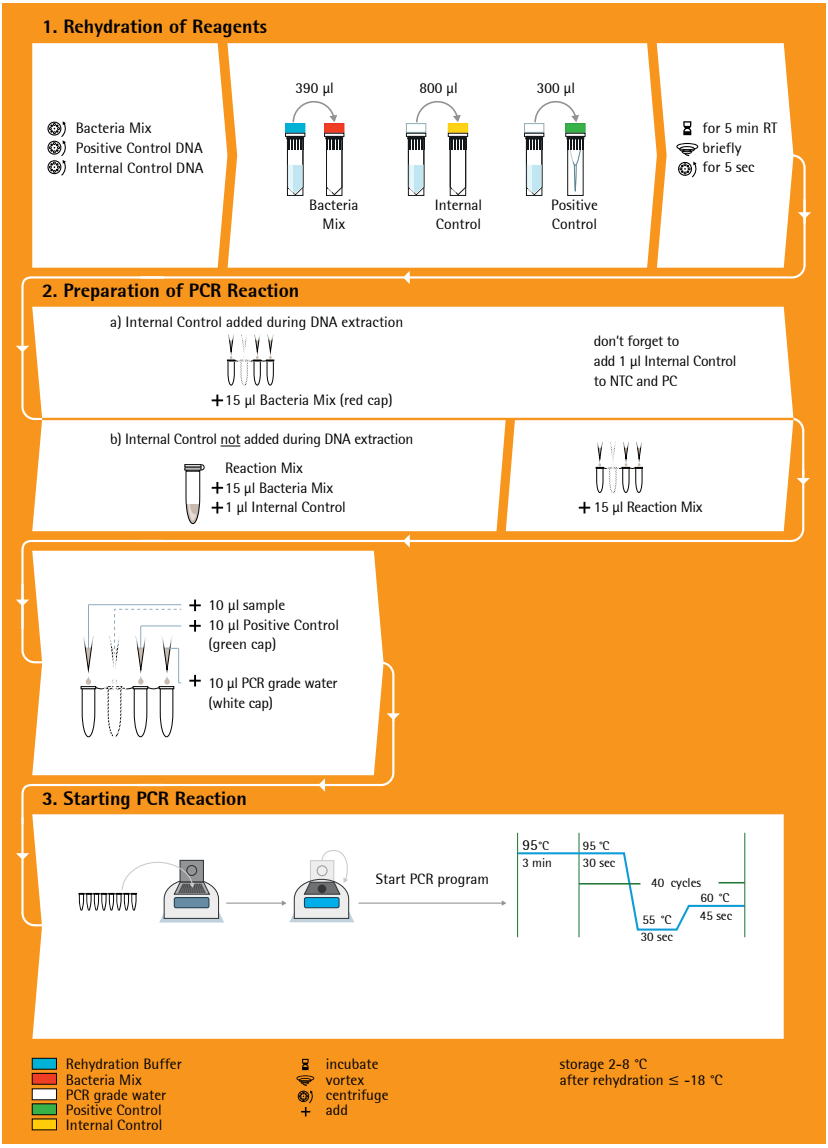
8.5 Starting the Reaction

1. Load the cycler, 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.
3. Start the program and data reading.

8.6 Analysis

1. Save the data at the end of the run.
2. Analyze the channels for the fluorescence dyes FAM™ and ROX™.
3. Adapt the threshold line to 10 % of the maximum fluorescence level of the positive controls (in case of double determination take the average of the maximum fluorescence levels). See chapter 11.
4. Analyze the calculation of the Ct-values for negative controls, positive controls and samples.

9. Short Instructions



10. Interpretation of Results

The presence of bacterial DNA in the sample is indicated by an increasing fluorescence signal in the FAM™ channel during PCR. The concentration of the contaminant can be calculated by a software comparing the crossing cycle number of the sample with a standard curve created in the same run.

A successfully performed PCR, without inhibition is indicated by an increasing fluorescence signal in the internal control channel. Bacterial 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 bacterial DNA loads in the sample.

10.1 Yes/No Evaluation

Detection of Bacteria FAM™ channel	Internal Control ROX™ channel	Interpretation
positive (Ct < 40)	irrelevant	Bacteria positive
negative (no Ct)	negative**	PCR inhibition *
negative (no Ct)	positive (Ct < 40)	Bacteria negative

*PCR inhibition might be caused by sample matrix. If one out of two replicates is negative for Internal Control (ROX™: no Ct), repeat the PCR. If two out of two replicates are negative for the Internal Control, extract DNA from your sample material and repeat the PCR.
** if used as PCR control, Internal control of bacteria negative samples (FAM™: no Ct) must show Ct-values in the range of +/- 2 cycles (ROX™) of the no-template control (master mix control). If used as process control, Internal Control of bacteria negative samples (FAM™: no Ct) must show Ct-values in the range of +/- 3 cycles (ROX™) of the no-template control (master mix control).

10.2 Total Analysis and recommended actions for product release testing

Sample	Expected Outcome	Unexpected Outcome	Action
NTC	negative	NTC positive	Repeat PCR only
PC	positive	PC negative	Repeat PCR only
NEC	negative	NEC positive	Repeat the whole process incl. DNA extraction and PCR
Specimen	0/2 positive		Product release
		1/2 positive	Repeat the whole process incl. DNA extraction and PCR New results: 0/2 positive product release 1/2 positive Low contamination 2/2 positive Contamination
		2/2 positive	Contamination

In case you want to identify a positive result, please send your PCR product to Minerva Biolabs GmbH. The PCR product will be purified by Minerva Biolabs. Sequencing will be done by an external sequencing service. The interpretation of your sequencing results will be supplied by Minerva Biolabs afterwards. Attention: in case of a light or multiple contamination, the sequencing analysis might lead to wrong identification.

Attention:
In case of a light or multiple contamination, the sequencing analysis might lead to wrong identification.

11. 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® ATMP Bacteria:

CFX96 Touch™ / CFX96 Touch Deep Well™
Mx3005P®
ABI Prism® 7500
RotorGene® 6000

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	40		
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

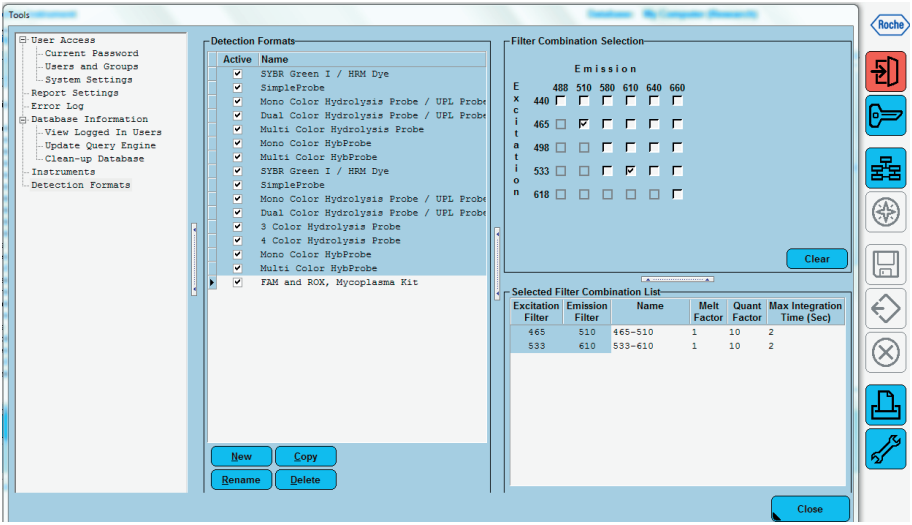
Analysis:
– Select the fluorescence channels Channel 1 (520 nm) and 3 (610 nm)
– Click on Quantification to generate the amplification plots and the specific Ct-values
– The threshold will be generated automatically
- Adapt the threshold line to 10 % of the maximum fluorescence level of the positive control (in case of duplicate determination, take the average of the maximum fluorescence levels)

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 "Bacteria Kit"
- Select the right filter combination by clicking the checkboxes with an 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	40		
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
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
Step Delay [Cycles]	0
Acquisition Mode	None

Data Analysis

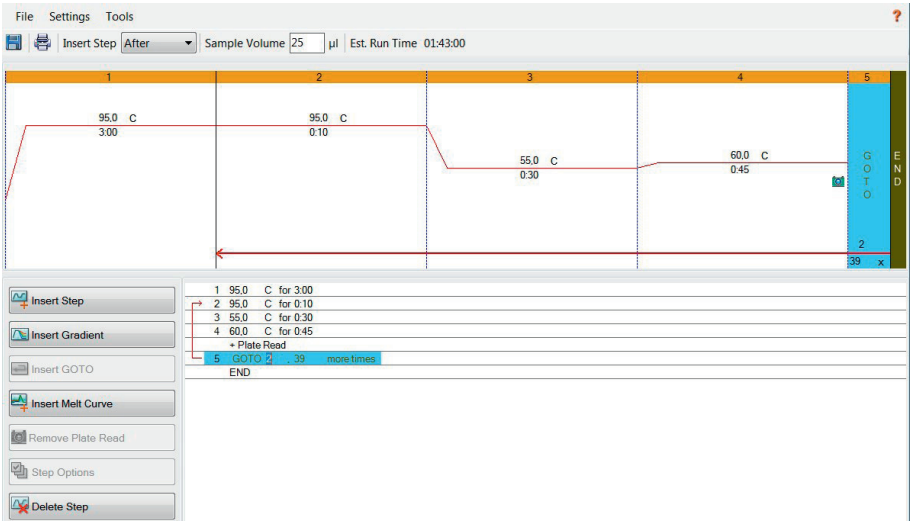
- Adapt the threshold line to 10 % of the maximum fluorescence level of the positive control (in case of duplicate determination, take the average of the maximum fluorescence levels)
- Select the Results tab to view specific Ct values

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

Run Setup Protocol Tab:

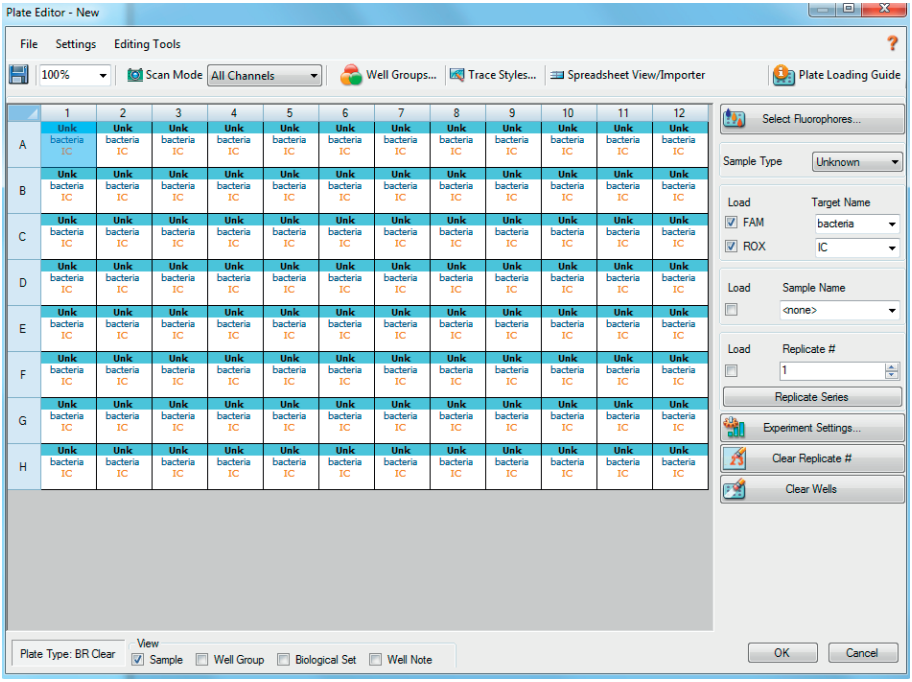
- Click File --> New --> Protocol to open the Protocol Editor and create a new protocol
- Select any step in either the graphical or text display
- Click the temperature or well time to directly edit the value

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
		GOTO Step 2, 39 more cycles		



Run Setup Plate Tab:

- Click File --> New --> Plate to open the Plate Editor and 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 bacteria 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 bacteria 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
- Remark: Amplification curves for which the baseline is not correctly calculated by the software, can be manually adapted
- By right-click 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
- Adapt the threshold line to 10 % of the maximum fluorescence level of the positive control (in case of duplicate determination, take the average of the maximum fluorescence levels)
- Evaluate the Ct-values according to chapter 10

RotorGene® 6000 (5-plex)

For the use of RotorGene® 6000, 0,1ml PCR tubes from Qiagen are recommended (Prod. No. 981106). Those tubes shall imperatively be used with the 72 well rotor from RotorGene® 6000.

1. Check the correct settings for the filter combination:

Target	Bacteria	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	40
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

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 10 % of the maximum fluorescence level of the positive control (in case of duplicate determination, take the average of the maximum fluorescence levels)
- 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	Bacteria	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 min

Program Step 2: Amplification

Cycles	40
Setting	Cycle
Denaturing	95 °C for 30 sec
Annealing	55 °C for 30 sec
Extension	60 °C for 45 sec

Analysis:

- Enter the following basic settings at the right task bar:
  Data: Delta RN vs. Cycle
  Detector: FAM™ and 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 10 % of the maximum fluorescence level of the positive control (in case of duplicate determination take the average of the maximum fluorescence levels)
- 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: 40 cycles	30 sec	95 °C
	30 sec	55 °C
	45 sec	60 °C data collection end

- 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“. The Threshold will be generated automatically
- Adapt the threshold line to 10 % of the maximum fluorescence level of the positive control (in case of duplicate determination take the average of the maximum fluorescence levels)
- Read the Ct-values in “Text Report“
- Evaluate the Ct-values according to chapter 10

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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Last technical revision: 2018-06-22

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
SMB95-1009	Microsart® RESEARCH Bacteria	25 tests
SMB95-1007	Microsart® ATMP Bacteria (patient)	10 patients

Microsart® Calibration Reagent, 1 vial, 10^8 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
SMB95-2030	Bacillus subtilis
SMB95-2031	Pseudomonas aeruginosa
SMB95-2032	Micrococcus luteus
SMB95-2033	Clostridium sporogenes
SMB95-2034	Bacteroides vulgatus
SMB95-2035	Staphylococcus aureus
SMB95-2036	Mycoplasma salivarium

Microsart® Validation Standard, 3 vials each, 10 CFU/vial for Mollicutes (SMB95-2011 – SMB95-2020) and 99 CFU/vial for other bacterial species (SMB95-2005-2010)

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
SMB95-2020	Mycoplasma salivarium
SMB95-2005	Bacillus subtilis
SMB95-2006	Pseudomonas aeruginosa

SMB95-2007	Micrococcus luteus	
SMB95-2008	Clostridium sporogenes	
SMB95-2009	Bacteroides vulgatus	
SMB95-2010	Staphylococcus aureus	

DNA Extraction Kits

SMB95-2001	Microsart® Bacteria Extraction	50 extractions
SMB95-2003	Microsart® AMP Extraction (only for Mycoplasma qPCR)	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 Wipes	120 wipes
15-2002	DNA Decontamination Wipes, refill sachets	5× 120 wipes

Mycoplasma Off™ *

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

Mycoplasma Off™ Wipes *

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

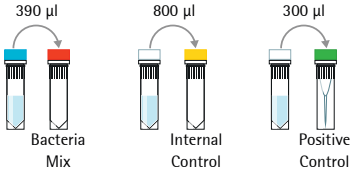
*** Distributed by Minerva Biolabs**

Short Instructions

Microsart® ATMP Bacteria

1. Rehydration of Reagents

- Bacteria Mix
- Positive Control DNA
- Internal Control DNA



- for 5 min RT
- briefly
- for 5 sec

2. Preparation of PCR Reaction

a) Internal Control added during DNA extraction

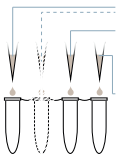
+ 15 µl Bacteria Mix (red cap)

don't forget to add 1 µl Internal Control to NTC and PC

b) Internal Control not added during DNA extraction

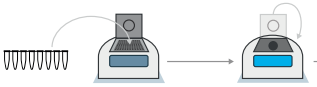
Reaction Mix
+ 15 µl Bacteria Mix
+ 1 µl Internal Control

+ 15 µl Reaction Mix

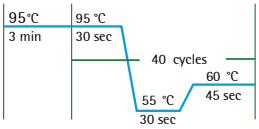


+ 10 µl sample
+ 10 µl Positive Control (green cap)
+ 10 µl PCR grade water (white cap)

3. Starting PCR Reaction



Start PCR program



- Rehydration Buffer
- Bacteria 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.

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GmbH, Goettingen, Germany

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Instructions for Use

Microsart® Bacteria Extraction

Bacterial DNA extraction kit

Prod. No. SMB95-2001

Reagents for 50 extractions

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 50 tests
	Manufacturer

Contents

1. Intended Use

Microsart® Bacteria Extraction kit introduces a state-of-the-art DNA extraction method for DNA extraction from cell cultures and cell culture derived biologicals, like autologous transplants and other advanced therapy medicinal products (ATMP), for subsequent DNA amplification via PCR.

2. Explanation of the Test

To achieve highest sensitivity and to avoid inhibitory effects in PCR testing, a DNA extraction is recommended. For most test materials, DNA extraction methods are available providing templates suitable for PCR. However, most of the DNA extraction kits available on the market are not free of bacterial DNA contaminations. Microsart® Bacteria Extraction introduces a unique DNA extraction method, which eliminates the risk of DNA contaminations, facilitating the detection of bacteria in cell culture and ATMPs via PCR.

The extraction procedure can be performed within 1 hour. In contrast to the culture method, samples do not need to contain vital bacteria, as all intact bacteria (e.g. live, dormant, non-culturable etc.) are detected.

3. Test Principle

Microsart® Bacteria Extraction kit was optimized for the extraction of genomic bacterial DNA from different sample matrices including cell culture samples. The contamination risk has been reduced to a minimum due to less handling steps.

An internal amplification control DNA from the Microsart® ATMP Bacteria kit can be added to the sample prior DNA extraction to monitor the extraction process by detecting false negative results which can occur due to improper DNA extraction or PCR inhibition. Alternatively, the internal control DNA can be added directly to the Mastermix during PCR setup.

4. Notes on the Test Procedure

1. For *in vitro* use in research and quality control. This kit may be disposed of according to local regulations.

2. This kit should be used only by trained persons. You should wear a clean lab coat and use disposable gloves at all times while performing the assay.

3. To avoid DNA cross-contaminations, the complete test must be performed under sterile and DNA-free conditions (see chapter 4.1 for detailed information).

4. In case of working with living bacteria strains, the local regulatory requirements for S2 labs must be considered.

5. Attention: by aliquoting and repeatedly freezing and thawing your samples, you run a high risk of contamination. This should therefore be avoided if possible.

6. This extraction kit has been validated with 1 ml starting volume. If you use less than 1 ml it must be ensured that 99 cfu can be detected in the appropriate volume.

7. This extraction kit is not suitable for the extraction of mycoplasma DNA. Therefore the DNA extract of this kit cannot be used for mycoplasma qPCR analysis.

8. This leaflet must be widely understood for a successful use of Microsart® Bacteria Extraction 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.

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

10. For each test setup, at least one negative extraction control should be included. Positive controls facilitate the evaluation of the test.

11. The controls should be carried out in the same manner as the samples.

4.1 Handling and Equipment Recommendations

To avoid false positive results due to improper handling the following actions are recommended:

1. To perform the test under sterile and DNA-free conditions, we recommend the use of an isolator/glovebox with an airlock.

2. The isolator/glovebox should be cleaned thoroughly with PCR Clean™ (Minerva Biolabs, Prod. No. 15-2025) or PCR Clean™ Wipes (Minerva Biolabs, Prod. No. 15-2001) before and during the working process.

3. All materials which are introduced into the isolator/glovebox should be cleaned thoroughly with PCR Clean™. Don´t forget to clean the airlock with PCR Clean™. Pipettes and gloves should be cleaned thoroughly with PCR Clean™ Wipes prior and during the process.

4. Avoid working above open tubes and avoid air turbulences due to rapid movements.

5. Be careful when opening the tubes. Do not touch the inner surface of the lid.

5. Reagents

Each kit contains reagents for 50 extractions. The expiry date of the unopened package is marked on the package label. The kit components are stored at ambient temperature until use. Suspension Buffer should be stored at 2 - 8 °C after first use.

Kit Component Label Information	50 Extractions Order No. SMB95-2001	Cap Color
Lysis Buffer	2 x 13 ml	transparent
Suspension Buffer	4 x 1.5 ml	violet
Processing Tubes	50 Tubes	

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

6. Needed but not Included

Microsart® Bacteria Extraction kit contains reagents for sample collection and DNA extraction. General industrial supplies and reagents, usually available in PCR laboratories are not included:

Consumables

- Laboratory gloves
- PCR Clean™ (Minerva Biolabs, Prod. No. 15-2025) and PCR Clean™ wipes (Minerva Biolabs, Prod. No. 15-2001)
- DNA-free pipette filter tips that must be free from bacterial DNA (we recommend Biosphere® filter tips from Sarstedt: 0.5-20 µl, No. 70.1116.210; 2-100 µl, Prod No. 70.760.212; 20-300 µl, Prod. No. 70.765.210; 100-1000 µl. Prod. No. 70.762.211)
- DNA-free PCR reaction tubes (PCR 8-SoftStrips with attached caps from Biozym are recommended: 0.1 ml Low Profile, Prod. No. 710975 and 0.2 ml High Profile, Prod. No. 710970)

Equipment

- Isolator/glovebox (further information, supplier and prices are available on request, please contact PCR@sartorius.com)
- Heat block
- Microcentrifuge for 1.5 ml reaction tubes (Centrisart A-14, Prod. No. A-14-1EU)
- Vortex Mixer
- Pipettes
  mechanical
  0.5 – 10 µl Sartorius Prod. No. LH-729020
  10 – 100 µl Sartorius Prod. No. LH-729050

100 – 1000 µl Sartorius Prod. No. LH-729070
or electrical
0,2 – 10 µl Sartorius Prod. No. 735021
10 – 300 µl Sartorius Prod. No. 735061
50 – 1000 µl Sartorius Prod No. 735081
- Rack for 1.5 ml tubes

For subsequent PCR analysis, the following consumables and equipment are required additionally:

- Bacterial DNA PCR detection system. We recommend the Microsart® ATMP Bacteria kit (Sartorius Prod. No. SMB95-1008), or the Microsart® RESEARCH Bacteria kit (Sartorius Prod. No. SMB95-1009/1010), both qPCR-based methods, designed for the direct detection of bacteria in cell cultures and cell culture derived biologicals

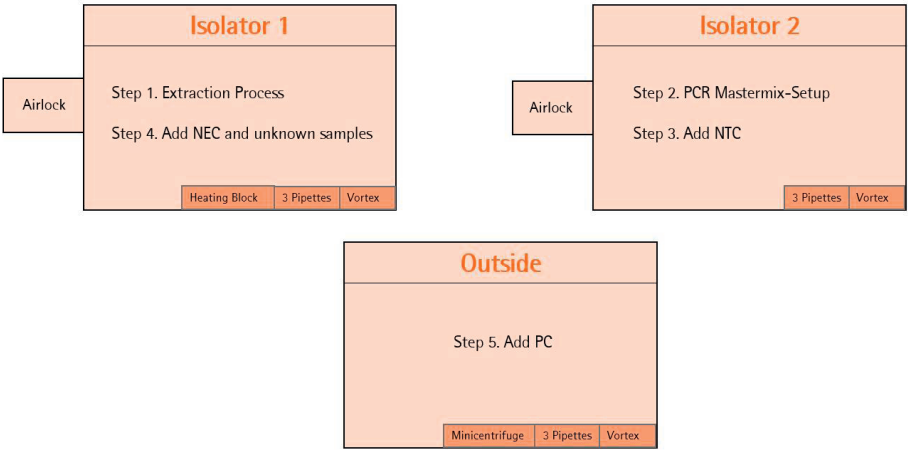
- qPCR device with filter sets for the detection of the fluorescence dyes FAM™ and ROX™ and suitable for 25 µl PCR reaction volumes

- DNA-free PCR reaction tubes that must be free from bacterial DNA for the specific qPCR device (PCR 8-SoftStrips with attached caps from Biozym are recommended: 0.1 ml Low Profile, Prod. No. 710975 and 0.2 ml High Profile, Prod. No. 710970)

- Minicentrifuge for PCR-tubes

- Isolator/glovebox (for PCR-setup)
- Vortex mixer
- Set of 3 pipettes
- Rack for 1.5 ml tubes
- Rack for PCR tubes

Schematical Overview of technical setup and experimental design

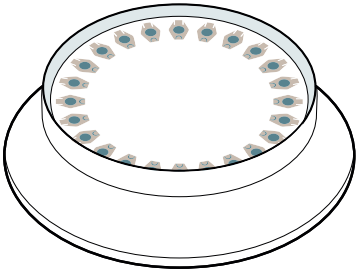


It is also possible to connect Isolator 1 and Isolator 2 via an airlock so that you can transfer your PCR-tubes after Step 3 directly from Isolator 2 into Isolator 1. Please note that you need an additional airlock for Isolator 2 in this case.

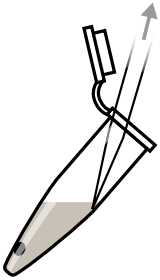
7. Specimen

Sample Collection and Storage

1. max. 1 ml liquid of cell culture or cell culture supernatant material is transferred into a provided DNA-free 1.5 ml processing tube (transparent cap).
   Attention: we recommend a maximum cell content of 10^6 cells/ml.
2. Spin down supernatant for 15 minutes at a speed of at least 16,200 x g to sediment bacteria particles.
   Attention: Make sure to position the tubes in the centrifuge in order to form the pellet on the back side of the tube, as explained on the figure below.
3. Discard the supernatant carefully and completely as explained on the figure below. Proceed to DNA Extraction. If DNA extraction cannot be performed immediately, freeze samples at ≤ -18 °C. Repeated freezing and thawing should be avoided.
   Attention: Samples can only be inactivated or frozen after this sample collection step.



Make sure to position the tubes with the back side toward the outside of the rotor in order to form the pellet on the back wall of the tube.



Slowly discard all the supernatant without disturbing the pellet.

8. Test Procedure

8.1 Recommendation for product release testing

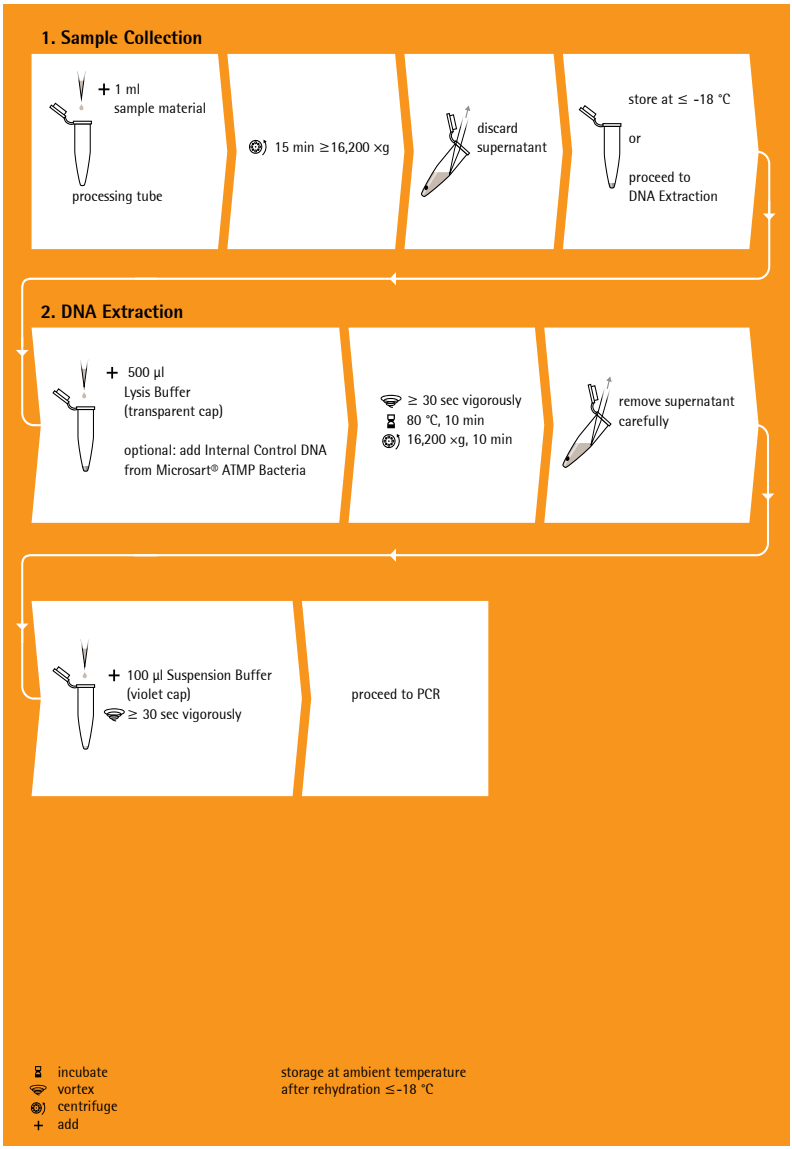
The extraction process should be carried out with a negative extraction control (NEC) and samples in duplicates (= 3 extractions for 1 product).

8.2 DNA Extraction Process

1.	Add 500 µl Lysis Buffer (transparent cap) to cell pellet. Recommended for users of Microsart® ATMP Bacteria detection kit: The Internal Control DNA which is included in the Microsart® ATMP Bacteria detection kit can also be used to monitor the extraction process. Add 20 µl Internal Control DNA to the sample, vortex briefly and proceed with step 2 as described. No additional Internal Control DNA is required for the PCR reaction mix.
2.	Vortex vigorously for at least 30 seconds until pellet is completely dissolved.
3.	Heat at 80°C for 10 minutes.
4.	Spin down at 16,200 x g for 10 minutes. Attention: make sure to position the tubes in the rotor as indicated on the figure Chapter 7.
5.	Remove supernatant carefully and completely following the explanations in paragraph 7. Make sure to not withdraw the pellet in the process. Attention: There is a high risk of inhibition in PCR analysis if residues remain in the tube.
6.	Add 100 µl Suspension Buffer (violet cap) and dissolve the DNA by thorough vortexing.

Extracts can be stored for 6 days at +2 to +8 °C. If long term storage is required, store at ≤ -18 °C. Repeated freezing and thawing should be avoided.

9. Short Instructions



10. Appendix

Limited Product Warranty
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Last technical revision: 2018-04-19

11. Related Products

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SMB95-1005/1006	Microsart® RESEARCH Mycoplasma	25/100 tests
SMB95-1009	Microsart® RESEARCH Bacteria	25 tests
SMB95-1008	Microsart® ATMP Bacteria	100 tests
SMB95-1007	Microsart® ATMP Bacteria (patient)	10 patients

Microsart® Calibration Reagent, 1 vial, 10^8 genomes / vial

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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
SMB95-2030	Bacillus subtilis
SMB95-2031	Pseudomonas aeruginosa
SMB95-2032	Micrococcus luteus
SMB95-2033	Clostridium sporogenes
SMB95-2034	Bacteroides vulgatus
SMB95-2035	Staphylococcus aureus
SMB95-2036	Mycoplasma salivarium

Microsart® Validation Standard, 3 vials each, 10 CFU/vial for Mollicutes (SMB95-2011 - SMB95-2020) and 99 CFU/vial for other bacterial species (SMB95-2005 - SMB95-2010)

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
SMB95-2005	Bacillus subtilis
SMB95-2006	Pseudomonas aeruginosa

SMB95-2007	Micrococcus luteus	
SMB95-2008	Clostridium sporogenes	
SMB95-2009	Bacteroides vulgatus	
SMB95-2010	Staphylococcus aureus	
SMB95-2020	Mycoplasma salivarium	
DNA Extraction Kit		
SMB95-2003	Microsart® AMP Extraction (only for Mycoplasma qPCR)	50 extractions
PCR Clean™ *		
15-2025	DNA Decontamination Reagent, spray bottle	250 ml
15-2200	DNA Decontamination Reagent, refill bottles	4x 500 ml
PCR Clean™ Wipes*		
15-2001	DNA Decontamination Wipes	120 wipes
15-2002	DNA Decontamination Wipes, refill sachets	5 x 120
Mycoplasma Off™ *		
15-1000	Surface Disinfectant Spray, spray bottle	1000 ml
15-5000	Surface Disinfectant Spray, refill bottles	5000 ml
Mycoplasma Off™ Wipes *		
15-1001	Surface Disinfectant Wipes	120 wipes
15-5001	Surface Disinfectant Wipes, refill sachets	5 x 120 wipes

*** Distributed by Minerva Biolabs**

Procedure-Overview

Microsart® Bacteria Extraction

1. Sample Collection

+ 1 ml
sample material

processing tube

15 min ≥16,200 ×g

discard
supernatant

store at ≤ -18 °C

or

proceed to
DNA Extraction

2. DNA Extraction

+ 500 µl
Lysis Buffer
(transparent cap)

optional: add Internal Control DNA
from Microsart® ATMP Bacteria

≥ 30 sec vigorously
80 °C, 10 min
16,200 ×g, 10 min

remove supernatant
carefully

+ 100 µl Suspension Buffer
(violet cap)
≥ 30 sec vigorously

proceed to PCR

incubate
vortex
centrifuge
+ add

storage at ambient temperature
after rehydration ≤-18 °C

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Instructions for Use

Microsart® ATMP Bacteria Patient

Bacteria DNA extraction and detection kit for qPCR

Prod. No. SMB95-1007

Reagents for 10 patients

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 50 tests
	Manufacturer

Contents

1. Intended Use

Microsart® ATMP Bacteria Patient kit is designed for the DNA extraction of bacteria in cell culture derived biologicals, like autologous transplants and ATMPs, and for the direct detection based on real-time PCR (qPCR). Be aware that this kit is not intended to be use as a diagnostic kit.

2. Explanation of the Test

Microsart® ATMP Bacteria Patient utilizes qPCR as the method of choice for sensitive and robust detection of bacterial contamination. To achieve highest sensitivity and to avoid inhibitory effects in PCR testing, DNA is previously extracted. Microsart® Bacteria Patient introduces a unique DNA extraction method, which eliminates the risk of DNA contaminations, facilitating the detection of bacteria in cell culture and ATMPs via PCR. The subsequent qPCR assay can be performed with any type of real-time PCR cycler able to detect the fluorescence dyes FAM™ and ROX™.

The complete detection procedure can be performed within 3.5 hours. In contrast to the culture method, samples do not need to contain vital bacteria as all intact bacteria (e.g. live, dormant, non-culturable etc.) are detected.

3. Test Principle

Microsart® ATMP Bacteria Patient kit was optimized for the extraction and detection of genomic bacterial DNA in samples derived from patients material including cell culture samples. The contamination risk has been reduced to a minimum due to less handling steps.

Bacteria are specifically detected by amplifying a highly conserved region of the rRNA operon, or more specifically, a fragment of the 16S rRNA coding region in the bacterial genome. The specific amplification is detected at 520 nm (FAM™ channel). The kit includes primer and FAM™ labeled probes which allow the specific detection of many bacterial species. The polymerase is part of the Bacteria Patient Mix. 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. The amplification of the internal amplification control is detected at 610 nm (ROX™ channel).

4. Notes on the Test Procedure

1. For *in vitro* use in research and quality control. This kit may be disposed of according to local regulations.

2. This leaflet must be widely understood for a successful use of Microsart® ATMP Bacteria Patient. 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.

3. This kit should be used only by trained persons. You should wear a clean lab coat and use disposable gloves at all times while performing the assay.

4. To avoid DNA cross-contaminations, the complete test must be performed under sterile and DNA-free conditions (see chapter 4.1 for detailed information).

5. In case of working with living bacteria strains, the local regulatory requirements for S2 labs must be considered.

6. This detection kit has been developed for 1 ml starting volume. If you use less than 1 ml it must be ensured that 99 cfu can be detected in the appropriate volume.

7. This kit is not suitable for the extraction of mycoplasma DNA. Therefore the DNA extract of this kit cannot be used for mycoplasma qPCR analysis.

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

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

10. The controls should be carried out in the same manner as the samples.

11. Inhibition of the qPCR may be caused by the sample matrix but also by sample elution buffer of DNA extraction kits which are not recommended. Do not use reagents from another kit than the Microsart® ATMP Bacteria Patient.

4.1 Handling and Equipment Recommendations

To avoid false positive results due to improper handling the following actions are recommended:

1. To perform the test under sterile and DNA-free conditions, we recommend the use of an isolator/glovebox with an airlock.

2. The isolator/glovebox should be cleaned thoroughly with PCR Clean™ (Minerva Biolabs, Prod. No. 15-2025) or PCR Clean™ Wipes (Minerva Biolabs, Prod. No. 15-2001) before and during the working process.

3. All materials which are introduced into the isolator/glovebox should be cleaned thoroughly with PCR Clean™. Don´t forget to clean the airlock with PCR Clean™. Pipettes and gloves should be cleaned thoroughly with PCR Clean™ Wipes prior and during the process.

4. Avoid working above open tubes and avoid air turbulences due to rapid movements.

5. Be careful when opening the tubes. Do not touch the inner surface of the lid.

5. Reagents

Each kit contains all reagents needed to test 10 patients. It consists of 10 individual patient tests containing material for three DNA extractions (sample in duplicate, 1x NEC) and five PCR reactions (2x sample, 1x NEC, 1x PC, 1x NTC). The expiry date of the unopened package is marked on the package label. The kit components are stored at 2-8 °C until use.

Kit Component Label Information	10 patients Order No. SMB95-1007	Cap Color
Lysis Buffer	10 x 1.8 ml	transparent
Suspension Buffer	10 x 1.5 ml	violet
Processing Tubes	10 x 3	
Bacteria Patient Mix	10 x lyophilized	red
Rehydration Buffer	10 x 0.5 ml	blue
Positive Control DNA	10 x lyophylized	green
Internal Control DNA	10 x lyophilized	yellow
PCR grade Water	20 x 1.5 ml	white

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

6. Needed but not Included

Microsart® Bacteria Patient kit contains reagents for DNA extraction and bacterial DNA detection. General industrial supplies and reagents, usually available in PCR laboratories are not included:

Consumables

- Laboratory gloves
- PCR Clean™ (Minerva Biolabs, Prod. No. 15-2025) and PCR Clean™ wipes (Minerva Biolabs, Prod. No. 15-2001)
- DNA-free pipette filter tips that must be free from bacterial DNA (Biosphere® filter tips from Sarstedt are recommended: 0.5-20 µl, Prod No. 70.1116.210; 2-100 µl, Prod No. 70.760.212; 20-300 µl, Prod. No. 70.765.210; 100-1000 µl. Prod.No. 70.762.211)
- DNA-free PCR reaction tubes (PCR 8-SoftStrips with attached caps from Biozym are recommended: 0.1 ml Low Profile, Prod. No. 710975 and 0.2 ml High Profile, Prod. No. 710970)

Equipment

- DNA-free PCR reaction tubes (PCR 8-SoftStrips with attached caps from Biozym are recommended: 0.1 ml Low Profile, Prod. No. 710975 and 0.2 ml High Profile, Prod. No. 710970)
- Isolator/glovebox (further information, supplier and prices are available on request, please contact PCR@sartorius.com)
- Heat block
- Microcentrifuge for 1.5 ml reaction tubes (Centrisart A-14, Prod. No. A-14-1EU)
- Vortex Mixer
- qPCR device with filter sets for the detection of the fluorescence dyes FAM™ and ROX™ and suitable for 25 µl PCR reaction volumes
- Minicentrifuge for PCR-tubes
- Pipettes (Sartorius)

  mechanical

  0.5 – 10 µl Sartorius Prod. No. LH-729020

  10 – 100 µl Sartorius Prod. No. LH-729050

  100 – 1000 µl Sartorius Prod. No. LH-729070

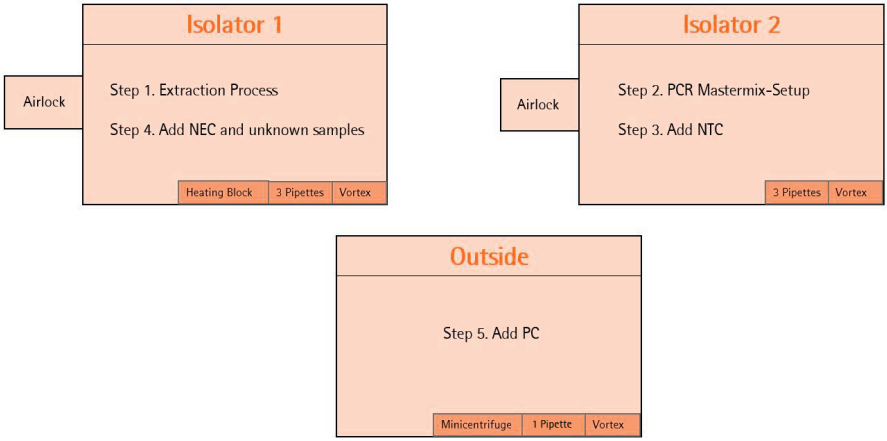
  or electrical

  0,2 – 10 µl Sartorius Prod. No. 735021

10 – 300 µl Sartorius Prod. No. 735061
50 – 1000 µl Sartorius Prod No. 735081

- Rack for 1,5 ml tubes and for PCR-tube strips

Schematical Overview of technical setup and experimental design



It is also possible to connect Isolator 1 and Isolator 2 via an airlock so that you can transfer your PCR-tubes after Step 3 directly from Isolator 2 into Isolator 1. Please note that you need an additional airlock for Isolator 2 in this case.

7. Test Procedure

7.1 Recommendation for product release testing

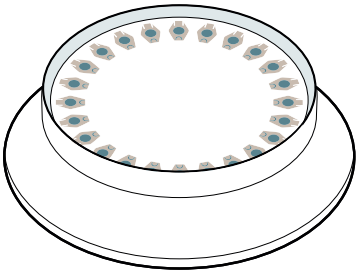
The extraction process should be carried out with a negative extraction control (NEC) and samples in duplicates (= 3 extractions for 1 product).

Additionally the PCR test should include a PCR negative control (NTC) and a PCR positive control.

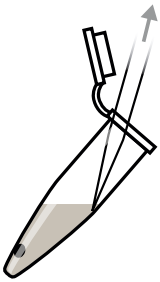
DNA extraction	PCR
2 x Sample	2 x Sample
1 x Negative Extraction Control	1 x Negative Extraction Control
	1 x PCR Positive Control
	1 x PCR Negative Control
∑ 3 extractions	∑ 5 PCR reactions

7.2 Sample Collection and Storage

1. max. 1 ml liquid of cell culture or cell culture supernatant material is transferred into a provided DNA-free 1.5 ml processing tube (transparent cap).
   Attention: we recommend a maximum cell content of 10^6 cells/ml.
2. Spin down for 15 minutes at a speed of at least 16,200 x g to sediment bacteria particles.
   Attention: Make sure to position the tubes in the centrifuge in order to form the pellet on the back side of the tube, as explained on the figure below.
3. Discard the supernatant carefully and completely as explained on the figure below. Proceed to DNA Extraction. If DNA extraction cannot be performed immediately, freeze samples at ≤ -18 °C. Repeated freezing and thawing should be avoided.
   Attention: Samples can only be inactivated or frozen after this sample collection step.



Make sure to position the tubes with the back side toward the outside of the rotor in order to form the pellet on the back wall of the tube.



Slowly discard all the supernatant without disturbing the pellet

7.3 DNA Extraction Process

1.	Add 500 µl Lysis Buffer (transparent cap) to cell pellet. Optional: The Internal Control DNA can also be used to monitor the extraction process. Add 20 µl Internal Control DNA to the sample, vortex briefly and proceed with step 2 as described. No additional Internal Control DNA is required for the PCR reaction mix.
2.	Vortex vigorously for at least 30 seconds until pellet is completely dissolved.
3.	Heat at 80°C for 10 minutes.
4.	Spin down at 16,200 x g for 10 minutes Attention: Make sure to position the tubes in the rotor as indicated on the figure Chapter 7.2.
5.	Remove supernatant carefully and completely following the explanations in paragraph 7.2. Make sure to not withdraw the pellet in the process. Attention: There is a high risk of inhibition in PCR analysis if residues remain in the tube.
6.	Add 100 µl Suspension Buffer (violet cap) and dissolve the DNA by thorough vortexing.

Extracts can be stored for 6 days at +2 to +8 °C. If long term storage is required, store at ≤ -18 °C. Repeated freezing and thawing should be avoided.

7.4 Rehydration of the Reagents

1.	Bacteria Patient Mix Internal Control DNA Positive Control	red cap yellow cap green cap	Centrifuge briefly
2.	Bacteria Patient Mix	red cap	Add 90 µl Rehydration Buffer (blue cap)
3.	Internal Control DNA	yellow cap	Add 800 µl PCR grade Water (white cap)
4.	Positive Control DNA	green cap	Add 300 µl PCR grade Water (white cap)
5.	Bacteria Patient Mix Internal Control DNA Positive Control DNA	red cap yellow cap green cap	Incubate 5 min at room temperature
6.	Bacteria Patient Mix Internal Control DNA Positive Control DNA	red cap yellow cap green cap	Vortex briefly

7.5 Preparation of the Reaction Mix

Preparation of the master mix and sample loading should not take more than 45 minutes to avoid a reduction in the fluorescent signal. The pipetting sequence should be respected and the tubes closed after each sample load.

The total volume per reaction is 25 µl including 10 µl sample.

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

1. Prepare the master mix at room temperature by addition of 6 µl Internal Control DNA (yellow cap) into the rehydrated Bacteria Patient Mix (red cap).
2. Homogenize the reaction mix by tapping carefully against the tube. Spin briefly.
3. Add 15 µl to each PCR tube. Close PCR tubes. Discard remaining liquid.

Attention:
If the Internal Control DNA was added to the sample during DNA extraction, add 15 µl of the Bacteria Patient Mix (red cap) directly to each PCR tube. Attention: Don´t forget to add 1µl of Internal Control DNA to NTC and PC.

7.6 Loading the Test Tubes

1. Negative controls: add 10 µl Suspension Buffer (violet cap) or PCR grade Water (white cap). Seal tube before proceeding with the samples.
   Attention: Negative controls should be processed in the isolator/glovebox used for mastermix setup.
2. Sample reaction: add 10 µl of sample. Seal tube tightly before proceeding.
   Attention: Samples, including NECs, should be added to the reaction in the isolator/glovebox used for DNA extraction.
3. Positive control: add 10 µl Positive Control DNA (green cap).
   Attention: Positive controls should not be handled in the isolator/glovebox used for mastermix setup or DNA extraction.
4. Close and spin all PCR tubes briefly, load the qPCR cycler and start the program.

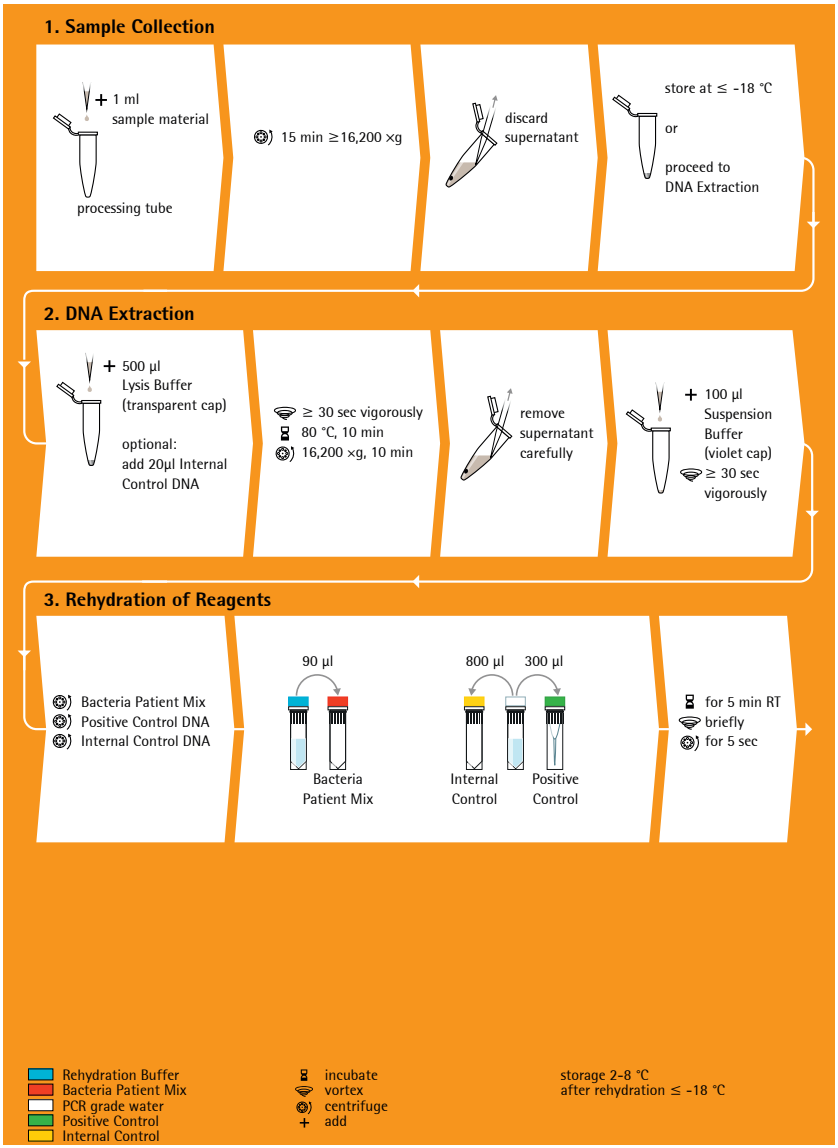
7.7 Starting the Reaction

1. Load the cycler, 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.
3. Start the program and data reading.

7.8 Analysis

1. Save the data at the end of the run.
2. Analyze the channels for the fluorescence dyes FAM™ and ROX™.
3. Adapt the threshold line to 10 % of the maximum fluorescence level of the positive controls (in case of double determination take the average of the maximum fluorescence levels). See chapter 10.
4. Analyze the calculation of the C_t-values for negative controls, positive controls and samples.

8. Short Instructions



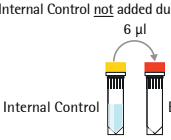
4. Preparation of PCR Reaction

a) Internal Control added during DNA extraction

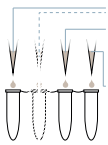
+ 15 µl Bacteria Patient Mix (red cap)

don't forget to
add 1 µl Internal Control
to NTC and PC

b) Internal Control not added during DNA extraction

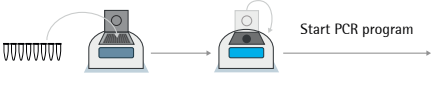


+ 15 µl Reaction Mix



+ 10 µl sample
+ 10 µl Positive Control (green cap)
+ 10 µl PCR grade water (white cap)

5. Starting PCR Reaction



9. Interpretation of Results

The presence of bacterial DNA in the sample is indicated by an increasing fluorescence signal in the FAM™ channel during PCR. The concentration of the contaminant can be calculated by a software comparing the crossing cycle number of the sample with a standard curve created in the same run.

A successfully performed PCR, without inhibition is indicated by an increasing fluorescence signal in the internal control channel. Bacterial 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 bacterial DNA loads in the sample.

9.1 Yes/No Evaluation

Detection of Bacteria FAM™ channel	Internal Control ROX™ channel	Interpretation	
positive (Ct < 40)	irrelevant	Bacteria positive	
negative (no Ct)	negative**	if used as PCR control	PCR inhibition
		if used as process control	Extraction or/and PCR inhibition
negative (no Ct)	positive (Ct < 40)	Bacteria negative	

*PCR inhibition might be caused by sample matrix. If one out of two Internal Control is negative (ROX™: no Ct), repeat the PCR. If two out of two Internal Control are negative, repeat the DNA extraction and the PCR.

** if used as PCR control, Internal control of bacteria negative samples (FAM™: no Ct) must show Ct-values in the range of +/- 2 cycles (ROX™) of the no-template control (master mix control). If used as process control, Internal Control of bacteria negative samples (FAM™: no Ct) must show Ct-values in the range of +/- 3 cycles (ROX™) of the no-template control (master mix control).

9.2 Total Analysis and recommended actions for product release testing

Sample	Expected Outcome	Unexpected Outcome	Action
NTC	negative	NTC positive	Repeat PCR only
PC	positive	PC negative	Repeat PCR only
NEC	negative	NEC positive	Repeat the whole process incl. DNA extraction and PCR
Specimen	0/2 positive		Product release
		1/2 positive	Repeat the whole process incl. DNA extraction and PCR New results: 0/2 positive product release 1/2 positive Low contamination 2/2 positive Contamination
		2/2 positive	Contamination

In case you want to identify a positive result, please send your PCR product to Minerva Biolabs GmbH. The PCR product will be purified by Minerva Biolabs. Sequencing will be done by an external sequencing service. The interpretation of your sequencing results will be supplied by Minerva Biolabs afterwards.

Attention:
In case of a light or multiple contamination, the sequencing analysis might lead to wrong identification.

10. 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™.

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	40		
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

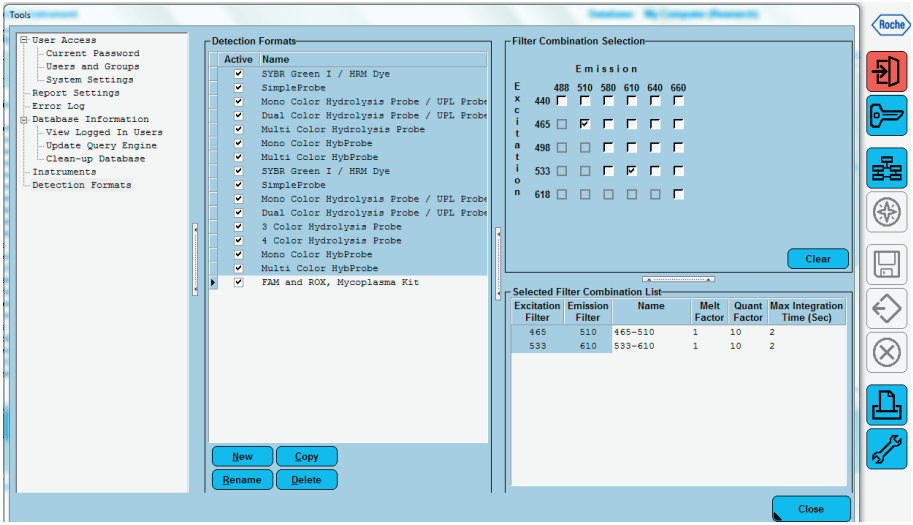
Analysis:
– Select the fluorescence channels Channel 1 (520 nm) and 3 (610 nm)
– Click on Quantification to generate the amplification plots and the specific Ct-values
– The threshold will be generated automatically
- Adapt the threshold line to 10 % of the maximum fluorescence level of the positive control (in case of duplicate determination, take the average of the maximum fluorescence levels)

LightCycler® 480 II

Choosing the correct filter setting:

- To define your filter combination, go to the Tool menu at the lower right 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 “Bacteria Kit”
- Select the right filter combination by clicking the checkboxes with an 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	40		
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
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
Step Delay [Cycles]	0
Acquisition Mode	None

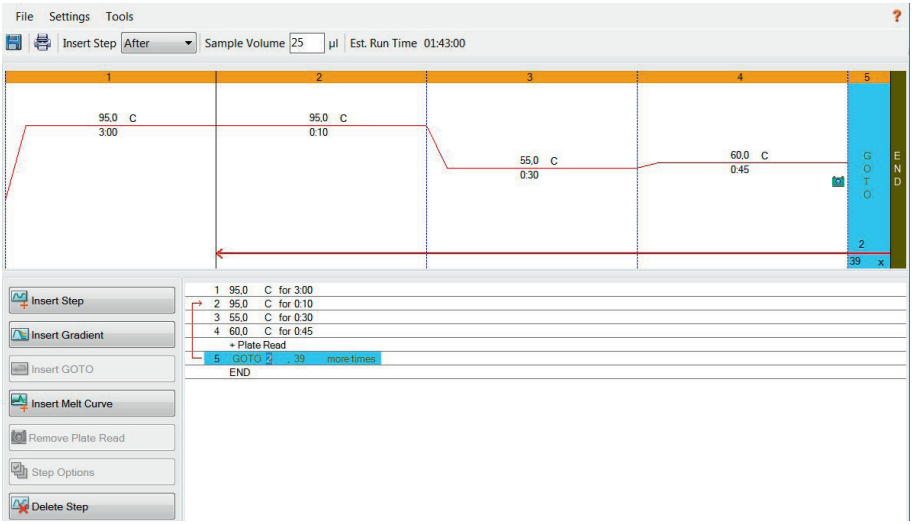
Data Analysis

- Adapt the threshold line to 10 % of the maximum fluorescence level of the positive control (in case of duplicate determination, take the average of the maximum fluorescence levels)
- Select the Results tab to view specific Ct values

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

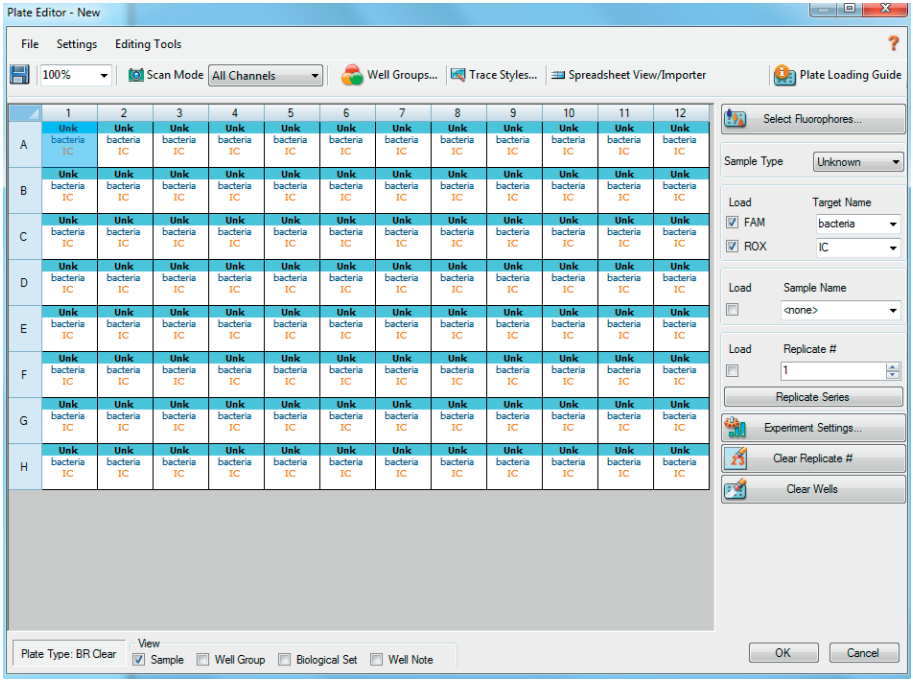
Run Setup Protocol Tab:
- Click File --> New --> Protocol to open the Protocol Editor and create a new protocol
- Select any step in either the graphical or text display
- Click the temperature or well time to directly edit the value

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
		GOTO Step 2, 39 more cycles		



Run Setup Plate Tab:

- 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 Type”
- 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 bacteria 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 bacteria 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
- Remark: Amplification curves for which the baseline is not correctly calculated by the software, can be manually adapted
- By right-click 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
- Adapt the threshold line to 10 % of the maximum fluorescence level of the positive control (in case of duplicate determination, take the average of the maximum fluorescence levels)
- Evaluate the Ct-values according to chapter 10

RotorGene® 6000 (5-plex)

For the use of RotorGene® 6000, 0,1ml PCR tubes from Qiagen are recommended (Prod. No. 981106). Those tubes shall imperatively be used with the 72 well rotor from RotorGene® 6000.

1. Check the correct settings for the filter combination:

Target	Bacteria	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	40
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

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 10 % of the maximum fluorescence level of the positive control (in case of duplicate determination, take the average of the maximum fluorescence levels)
- 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	Bacteria	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 min

Program Step 2: Amplification

Cycles	40
Setting	Cycle
Denaturing	95 °C for 30 sec
Annealing	55 °C for 30 sec
Extension	60 °C for 45 sec

Analysis:

- Enter the following basic settings at the right task bar:
  Data: Delta RN vs. Cycle
  Detector: FAM™ and 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 10 % of the maximum fluorescence level of the positive control (in case of duplicate determination take the average of the maximum fluorescence levels)
- 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: 40 cycles	30 sec	95 °C
	30 sec	55 °C
	45 sec	60 °C data collection end

- 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". The Threshold will be generated automatically
- Adapt the threshold line to 10 % of the maximum fluorescence level of the positive control (in case of duplicate determination, take the average of the maximum fluorescence levels)
- Read the Ct-values in "Text Report"
- Evaluate the Ct-values according to chapter 10

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.

Trademarks

Microsart is a registered trademark of Sartorius Stedim Biotech. Myocoplasma Off and PCR Clean are trademarks of Minerva Biolabs GmbH, Germany.

Last technical revision: 2018-04-20

11. 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
SMB95-1009	Microsart® RESEARCH Bacteria	25 tests
SMB95-1008	Microsart® ATMP Bacteria	100 tests
SMB95-1007	Microsart® ATMP Bacteria (patient)	10 patients

Microsart® Calibration Reagent, 1 vial, 10^8 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
SMB95-2030	Bacillus subtilis
SMB95-2031	Pseudomonas aeruginosa
SMB95-2032	Micrococcus luteus
SMB95-2033	Clostridium sporogenes
SMB95-2034	Bacteroides vulgatus
SMB95-2035	Staphylococcus aureus
SMB95-2036	Mycoplasma salivarium

Microsart® Validation Standard, 3 vials each, 10 CFU/vial for Mollicutes (SMB95-2011 - SM95-2020) and 99 CFU/vial for other bacterial species (SMB95-2005 - SMB95-2010)

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
SMB95-2020	Mycoplasma salivarium
SMB95-2005	Bacillus subtilis

SMB95-2006	Pseudomonas aeruginosa	
SMB95-2007	Micrococcus luteus	
SMB95-2008	Clostridium sporogenes	
SMB95-2009	Bacteroides vulgatus	
SMB95-2010	Staphylococcus aureus	

DNA Extraction Kit

SMB95-2003	Microsart® AMP Extraction (only for Mycoplasma qPCR)	50 extractions

PCR Clean™ *

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

PCR Clean™ Wipes*

15-2001	DNA Decontamination Wipes	120 wipes
15-2002	DNA Decontamination Wipes, refill sachets	5 x 120

Mycoplasma Off™ *

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

Mycoplasma Off™ Wipes *

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

*** Distributed by Minerva Biolabs**

Short Instructions

Microsart® Bacteria Patient

1. Sample Collection

+ 1 ml sample material

processing tube

15 min ≥16,200 ×g

discard supernatant

store at ≤ -18 °C

or

proceed to DNA Extraction

2. DNA Extraction

+ 500 µl Lysis Buffer (transparent cap)

optional: add 20µl Internal Control DNA

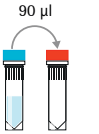
≥ 30 sec vigorously
80 °C, 10 min
16,200 ×g, 10 min

remove supernatant carefully

+ 100 µl Suspension Buffer (violet cap)
≥ 30 sec vigorously

3. Rehydration of Reagents

Bacteria Patient Mix
Positive Control DNA
Internal Control DNA



for 5 min RT
briefly
for 5 sec

Rehydration Buffer
Bacteria Patient Mix
PCR grade water
Positive Control
Internal Control

incubate
vortex
centrifuge
+ add

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

4. Preparation of PCR Reaction

a) Internal Control added during DNA extraction

+ 15 µl Bacteria Patient Mix (red cap)

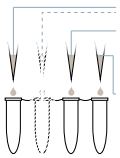
don't forget to
add 1 µl Internal Control
to NTC and PC

b) Internal Control not added during DNA extraction

6 µl

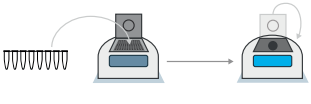
Internal Control Bacteria Patient Mix

+ 15 µl Reaction Mix

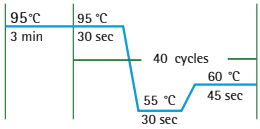


+ 10 µl sample
+ 10 µl Positive Control (green cap)
+ 10 µl PCR grade water (white cap)

5. Starting PCR Reaction



Start PCR program



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Ver.06 | 2018

Instructions for Use

Microsart® Calibration Reagent

Prod. No. SMB95-2021 Mycoplasma arginini
Prod. No. SMB95-2022 Mycoplasma orale
Prod. No. SMB95-2023 Mycoplasma gallisepticum
Prod. No. SMB95-2024 Mycoplasma pneumoniae
Prod. No. SMB95-2025 Mycoplasma synoviae
Prod. No. SMB95-2026 Mycoplasma fermentans
Prod. No. SMB95-2027 Mycoplasma hyorhinis
Prod. No. SMB95-2028 Acholeplasma laidlawii
Prod. No. SMB95-2029 Spiroplasma citri
Prod. No. SMB95-2030 Bacillus subtilis
Prod. No. SMB95-2031 Pseudomonas aeruginosa
Prod. No. SMB95-2032 Kocuria rhizophila
Prod. No. SMB95-2033 Clostridium sporogenes
Prod. No. SMB95-2034 Bacteroides vulgatus
Prod. No. SMB95-2035 Staphylococcus aureus
Prod. No. SMB95-2036 Mycoplasma salivarium

For use in research and quality control

Symbols

LOT	Lot No.
REF	Order No.
	Expiry date
	Store at
	Content

Contents

1. Intended Use

Titrated genomic DNA can be used as amplification and sensitivity control of end point PCR (gel-based evaluation). For quantitative PCR, titrated genomic DNA can be used to create standard curves by performing dilution series of the material as template for PCR. The software of various devices will be able to calculate from qPCR data corresponding concentrations and will generate a standard curve, which can be used to determine unknown DNA concentrations.

2. Explanation of the Product

This product provides isolated genomic DNA of a specific species. To cultivate the microorganisms a culture medium was inoculated with an early passage strain and harvested at the end of the logarithmic growth phase by repeated washing and centrifugation. Following the extraction, the DNA concentration was quantified photometrically (OD260/280) and with an ultrasensitive fluorescent nucleic acid stain for quantifying double-stranded DNA (dsDNA) calibrated to weight reference standards and controlled by qPCR (compared against exactly quantified calibrator plasmids). The DNA concentration was adjusted with regular TE80 buffer.

The following species are used

Mycoplasma arginini	ATCC 23838; NCTC 10129
Mycoplasma orale	ATCC 23714; NCTC 10112; DSM 25590
Mycoplasma gallisepticum	ATCC 19610; NCTC 10115; DSM 19817
Mycoplasma pneumoniae	ATCC 15531; NCTC 10119; DSM 22911
Mycoplasma synoviae	ATCC 25204; NCTC 10124; DSM 21430
Mycoplasma fermentans	ATCC 19989; NCTC 10117
Mycoplasma hyorhinis	ATCC 17981; NCTC 10130; DSM 25591
Acholeplasma laidlawii	ATCC 23206; NCTC 10116; DSM 23060
Spiroplasma citri	ATCC 27556; NCTC 10164; DSM 21846
Bacillus subtilis	ATCC 6633; NCTC 10400; DSM 347
Pseudomonas aeruginosa	ATCC 9027; DSM 1128
Kocuria rhizophila	ATCC 9341; DSM 348
Clostridium sporogenes	ATCC 19404; NCTC 532; DSM 1664
Bacteroides vulgatus	ATCC 8482; DSM 1447
Staphylococcus aureus	ATCC 6538; NCTC 10788; DSM 799

3. Principle

Each vial contains $1x10^8$ copies of the complete bacterial genome. The material cannot be used for cultivation methods. The genome standard can directly be used for PCR.
Please note: European Pharmacopoeia does not provide sensitivity limits on DNA level. For process validation use Microsart® Validation Standards.

4. Reagents

Each kit contains 1 vial of bacterial DNA ($1x10^8$ genome copies) and 3 vials of buffer for the preparation of dilutions and negative controls. All samples are lyophilized for product stability reasons. The material has been inactivated prior lyophilisation and can be considered as non-infectious. The expiry date of the unopened product is specified on the package label. The kits components are stored until use at +2 to +8 °C and must be stored at ≤-18 °C after rehydration.

Kit Component Label Information	Quantity	Cap Color
Calibration Reagent	1 × lyophilized	green
Buffer	3	white

5. Needed but not Included

Microsart® Calibration Reagent contains the positive and negative material to perform the test. General industrial supplies and reagents, usually available in PCR laboratories are not included:

Consumables

- Laboratory gloves
- DNA-free pipette filter tips that must be free from bacterial DNA (Biosphere® filter tips from Sarstedt are recommended: 0.5-20 µl, Prod No. 70.1116.210; 2-100 µl, Prod No. 70.760.212; 20-300 µl, Prod. No. 70.765.210; 100-1000 µl. Prod. No. 70.762.211)

Equipment

- Microcentrifuge for 1.5 ml reaction tubes (Centrisart A-14, Prod. No. A-14-1EU)
- Vortex
- Pipettes

mechanical	0.5 – 10 µl Sartorius Prod. No. LH-729020
	10 – 100 µl Sartorius Prod. No. LH-729050
	100 – 1000 µl Sartorius Prod. No. LH-729070
or electrical	0,2 – 10 µl Sartorius Prod. No. 735021
	10 – 300 µl Sartorius Prod. No. 735061
	50 – 1000 µl Sartorius Prod No. 73508

- Rack for 1.5 ml tubes

For PCR analysis, a bacteria or mycoplasma DNA PCR detection system is required.

- For Bacterial DNA we recommend the Microsart® ATMP Bacteria kit (Sartorius Prod. No. SMB95-1008), or the Microsart® RESEARCH Bacteria kit (Sartorius Prod. No. SMB95-1009).
- For detection of mycoplasma DNA we recommend the Microsart® ATMP Mycoplasma (Sartorius Prod. No. SMB95-1003/1004); Microsart® AMP Mycoplasma (Sartorius Prod. No. SMB95-1001/1002) or Microsart® RESEARCH Mycoplasma (Sartorius Prod. No. SMB95-1005/1006)

6. Precautions

For *in vitro* use in research and quality control. This kit should be used only by trained persons. This kit does not contain hazardous substances and may be disposed of according to local regulations.

7. Procedure

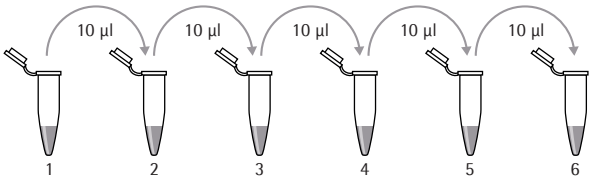
7.1 Rehydration of the reagents

All reagents must be equilibrated to room temperature prior use.

1. Centrifuge the tube briefly to collect the lyophilized material at the bottom of the tube.
2. Add 100 µl Buffer (white cap) to the vial containing the Calibration Reagent (green cap) to obtain a concentration of $1x10^6$ genomes/ µl.
3. Incubate 5 min at room temperature.
4. Vortex for 10 sec. and spin for 5 sec. with the "pulse" option or at 5000 g.
5. Aliquots the vial content in DNA-free tubes and freeze ≤ -18 °C or directly proceed to step 7.2.

7.2 Preparation of the Dilutions

1. Thaw the resuspended Calibration Reagent if applicable.
2. Label six 1.5 ml reaction tubes consecutively and fill each with 90 µl of Buffer (white cap).
3. Vortex Calibration Reagent briefly (1 to 2 seconds) at medium speed.
4. Add 10 µl of the Calibration Reagent to reaction tube no. 1, close the tube and vortex briefly at medium speed.
5. Add 10 µl of the content of reaction tube no. 1 to reaction tube no. 2.
6. Close the tube and vortex briefly at medium speed.
7. Proceed with the following reaction tubes of the dilution series in the same way.



7.3 PCR

Please follow the PCR kit manual. The volume used as template for PCR defines the number of genome copies per reaction:

Reaction tube	2 µl sample volume	10 µl sample volume	50 µl sample volume
1.	$2x10^5$ genome copies	$1x10^6$ genome copies	$5x10^6$ genome copies
2.	$2x10^4$ genome copies	$1x10^5$ genome copies	$5x10^5$ genome copies
3.	$2x10^3$ genome copies	$1x10^4$ genome copies	$5x10^4$ genome copies
4.	200 genome copies	1000 genome copies	5000 genome copies
5.	20 genome copies	100 genome copies	500 genome copies
6.	2 genome copies	10 genome copies	50 genome copies

7.4 Evaluation

In qPCR the Ct-values should decrease linearly with ascending DNA concentration per reaction, when a suitable PCR assay is used. The software of the qPCR device calculates a standard curve and slope using the DNA concentrations stated by the user and the appendant Ct-values. Also, the Ct-values of samples with unknown DNA concentrations are automatically compared to the standard curve, and concentrations are assigned. The following figures were generated using the Mx 3005p instrument.

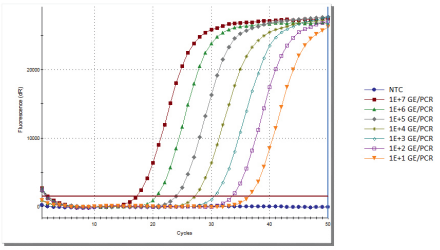


Fig. 1: Amplification curves of a dilution series from $1x10^7$ to 10 genome copies/reaction.

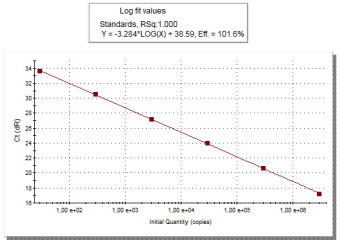


Fig. 2: Standard curve generated with the Mx 3005p instrument using second derivative maximum method and the data from Fig. 1

8. Notes on the Procedure

1. This leaflet must be widely understood for a successful use of the Microsart® Calibration Reagent. The reagents supplied should not be mixed with reagents from different lots and used as an integral unit. The reagents of the kit should not be used beyond their shelf life.
2. Any deviation from the described method can affect the results.
3. For each test setup, at least one negative control should be added.
4. Participation in external quality control programs, such as offered by Minerva Biolabs GmbH (www.minerva-biolabs.com) on an biannual base, is recommended.

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 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.

Trademarks
Microsart is a registered trademark of Sartorius Stedim Biotech GmbH. Mycoplasma Off and PCR Clean are a trademark of Minerva Biolabs.

Last technical revision: 2018-07-10

9. 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
SMB95-1007	Microsart® ATMP Bacteria Patient	10 patients
SMB95-1008	Microsart® ATMP Bacteria	100 tests
SMB95-1009	Microsart® RESEARCH Bacteria	25 tests

Microsart® Validation Standard

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
SMB95-2020	Mycoplasma salivarium
SMB95-2005	Bacillus subtilis
SMB95-2006	Pseudomonas aeruginosa
SMB95-2007	Kocuria rhizophila
SMB95-2008	Clostridium sporogenes
SMB95-2009	Bacteroides vulgatus
SMB95-2010	Staphylococcus aureus

DNA Extraction Kit

SMB95-2001	Microsart® Bacteria Extraction	50 extractions
SMB95-2003	Microsart® AMP Extraction (only for Mycoplasma qPCR)	50 extractions

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

UNG Carry over prevention*

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

PCR Clean™ (formerly DNA Remover™) *

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

PCR Clean™ Wipes*

15-2001	DNA Decontamination Wipes	120 wipes
15-2002	DNA Decontamination Wipes, refill sachets	5x 120 wipes

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**

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The status of the information,
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the date given below.
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specifications and design of the
equipment without notice.

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Order No.: 85037-546-77
Ver. 09 | 2018

Instructions for Use

Microsart® RESEARCH Bacteria

Bacteria Detection Kit for qPCR

Prod. No. SMB95-1009

Reagents for 25 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 tests
	Manufacturer

Contents

1. Intended Use

Microsart® RESEARCH Bacteria is used for direct detection of bacterial contamination in cell cultures and cell media components in research and development.

2. Explanation of the Test

Microsart® RESEARCH Bacteria utilizes real-time PCR (qPCR). 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 fast and reliable screening of cell culture supernatants most applicable in research and development. The detection procedure can be performed within three hours. In contrast to the culture method, samples do not need to contain vital bacteria.

3. Test Principle

Bacteria are specifically detected by amplifying a highly conserved rRNA operon, or more specifically, a 16S rRNA coding region in the bacterial genome. The amplification is detected at 520 nm (FAM™ channel). The kit includes primer and FAM™ labeled probes which allow the specific detection of many bacterial 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 Bacteria RESEARCH Mix. The amplification of the internal amplification control is detected at 610 nm (ROX™ channel).

4. Notes on the Test Procedure

1. For in vitro use in research. This kit may be disposed of according to local regulations.

2. This kit should be used only by trained persons. You should wear a clean lab coat and use disposable gloves at all times while performing the assay.

3. To avoid DNA cross-contaminations, the complete test must be performed under sterile and DNA-free conditions.

4. Always use a new unopened DNA-free pipette filter tip-box for each assay. Reaction vials should always be closed immediately after every pipetting step.

5. It is recommended to perform the assay in a predecontaminated, UV-treated laminar flow cabinet. Spatial segregation of the sequential steps is recommended.

6. In case of working with living bacteria strains, the local regulatory requirements for S2 labs must be considered.

7. Attention: by aliquoting and freezing your samples you run a high risk of contamination. This should therefore be avoided if possible.

8. This leaflet must be widely understood for a successful use of Microsart® RESEARCH Bacteria. 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.

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

10. Inhibition may be caused by the sample matrix, but also by sample elution buffer of DNA extraction kits which are not recommended or validated. Please note that by using DNA extraction kits which are not validated you run a high risk of obtaining false-positive or false-negative results.

11. 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.

12. The use of control samples is advised to secure the day-to-day validity of results. The controls should be carried out in the same manner as the samples.

4.1 Handling and Equipment Recommendations

1. The clean bench should be cleaned thoroughly with PCR Clean™ (Minerva Biolabs, Prod. No. 15-2025) or PCR Clean™ Wipes (Minerva Biolabs, Prod. No. 15-2001) before use.

2. All materials which are introduced into the clean bench should be cleaned thoroughly with PCR Clean™ prior the process.

3. Avoid working above open tubes and avoid air turbulences due to rapid movements.

4. Be careful when opening the tubes. Do not touch the inner surface of the lid.

5. Reagents

Each kit contains reagents for 25 reactions. The expiry date of the unopened package is marked on the package label. The kit components are stored until use at +2 to +8 °C and must be stored at ≤ -18 °C after rehydration. Protect the Bacteria RESEARCH Mix from light.

Kit Component Label Information	Quantity 25 Reactions Order No. SMB95-1009	Cap Color
Bacteria RESEARCH Mix	1 × lyophilized	red
Rehydration Buffer	1 × 1.0 ml	blue
Positive Control DNA	1 × lyophilized	green
PCR grade Water	1 × 1.5 ml	white

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

6. Needed but not Included

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

Consumables

- Laboratory gloves
- PCR Clean™ (Minerva Biolabs, Prod. No. 15-2025) and PCR Clean™ wipes (Minerva Biolabs, Prod. No. 15-2001)
- DNA-free PCR reaction tubes (PCR 8-SoftStrips with attached caps from Biozym are recommended: 0.1 ml Low Profile, Prod. No. 710975 and 0.2 ml High Profile, Prod. No. 710970)
- DNA-free pipette filter tips that must be free from bacterial DNA (Biosphere® filter tips from Sarstedt are recommended: 0.5-20 µl, Prod No. 70.1116.210; 2-100 µl, Prod No. 70.760.212; 20-300 µl, Prod. No. 70.765.210; 100-1000 µl. Prod. No. 70.762.211)
- Optional: Microsart® Bacteria Extraction kit, a DNA-free extraction kit, Sartorius Prod. No. SMB95-2001.

Equipment

- qPCR device with filter sets for the detection of the fluorescence dyes FAM™ and ROX™ and suitable for 25 µl PCR reaction volumes
- Minicentrifuge for 2ml reaction tubes and PCR-tubes
- Vortex mixer
- Heat-block
- Pipettes (Sartorius)

mechanical	0.5 – 10 µl Sartorius Prod. No. LH-729020
	10 – 100 µl Sartorius Prod. No. LH-729050
	100 – 1000 µl Sartorius Prod. No. LH-729070
or electrical	0,2 – 10 µl Sartorius Prod. No. 735021
	10 – 300 µl Sartorius Prod. No. 735061
	50 – 1000 µl Sartorius Prod. No. 735081

7. Specimen

Microsart® RESEARCH Bacteria does not require DNA extraction prior use. Samples can be obtained directly from cell culture supernatants.

Samples directly received from cell cultures contain DNases which can degrade bacterial DNA even at lower temperatures. If the test cannot be performed immediately after sampling, samples should be stabilized by heat inactivation at 95 °C for 10 min and stored at ≤ - 18 °C until use.

1. Transfer up to 500 µl of cell culture supernatant or cell culture material with up to 10^6 cells/ml to a sterile micro centrifuge tube. The lid should be tightly sealed to prevent opening during heating.
2. Incubate the sample at 95 °C for 10 minutes.
3. Briefly centrifuge (5 seconds) the sample at approx. 13,000 x g to pellet cellular debris.
4. The supernatant is used for PCR analysis.

If you detect any inhibitory effects, DNA extraction i.e. with Microsart® Bacteria Extraction (Prod No. SMB95-2001) is mandatory. 2 µl of the extract can be used directly as PCR template.

Repeated freezing and thawing of samples should be avoided.

8. Test Procedure

The test should be carried out with negative and positive controls and samples in duplicates. For quantification, set up a dilution series of an appropriate standard. For this, we recommend Microsart® Calibration Reagents (see Related Products for ordering information). 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 must be stored in aliquots.

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

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 respected and the tubes closed after each sample load.

1. Homogenize the rehydrated Bacteria RESEARCH Mix by vortexing. Spin briefly.
2. Add 23 µl to each PCR tube.
3. Negative control: Add 2 µl PCR grade Water (white cap)
4. Sample reaction: Add 2 µl of sample.
5. Positive control: Add 2 µl Positive Control DNA (green cap).
6. Close and spin all PCR tubes briefly, load the qPCR cycler and start the program.

8.3 Starting the reaction

1. Load the cycler, 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.
3. Start the program and data reading.

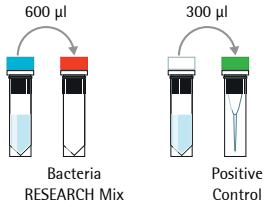
8.4 Analysis

1. Save the data at the end of the run.
2. Show amplification plots for FAM™ and ROX™ in linear mode.
3. Adapt the threshold line to 10 % of the maximum fluorescence level of the positive controls (in case of double determination take the average of the maximum fluorescence levels) (See chapter 11).
4. Read the calculation of the Ct-values for the negative controls, the positive controls and the samples.

9. Short Instructions

1. Rehydration of Reagents

Bacteria RESEARCH Mix and Positive Control

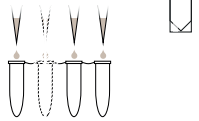


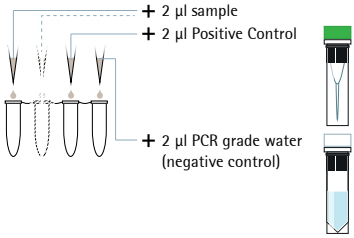
for 5 min RT
briefly
for 5 sec

2. Preparation of PCR Reactions

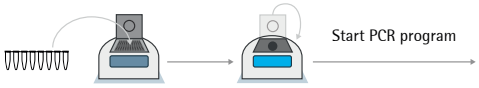
loading the test tubes

+ 23 µl Bacteria RESEARCH Mix

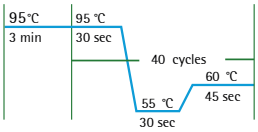




3. Starting the PCR Reaction



Start PCR program



Rehydration Buffer
Bacteria RESEARCH Mix
PCR grade water
Positive Control

incubate
vortex
centrifuge
add

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

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

10. Interpretation of Results

The presence of bacterial DNA in the sample is indicated by an increasing fluorescence signal in the FAM™ channel during PCR. The concentration of the contaminant can be calculated by a software comparing the crossing cycle number of the sample with a standard curve created in the same run.

A successfully performed PCR without inhibition is indicated by an increasing fluorescence signal in the internal control channel (ROX™). Bacterial 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 bacteria DNA loads in the sample.

10.1 Yes/No Evaluation

Detection of Bacteria FAM™ channel	Internal Control ROX™ channel	Interpretation
positive (Ct < 40)	irrelevant	Bacteria positive
negative (no Ct)	negative **	PCR inhibition *
negative (no Ct)	positive (Ct < 40)	Bacteria negative

* PCR inhibition might be caused by sample matrix. If one out of two replicates is negative for Internal Control (ROX™: No Ct), repeat the PCR. If two out of two replicates are negative for the Internal Control, extract DNA from your sample material and repeat the PCR.
** Internal control of bacteria negative samples (FAM™: no Ct) must show Ct-values in the range of +/- 2 cycles (ROX™) of the no-template control (master mix control).

10.2 Total Analysis and recommended actions

Sample	Expected Outcome	Unexpected Outcome	Action
NTC	negative	NTC positive	Repeat PCR
PC	positive	PC negative	Repeat PCR
Specimen	0/2 positive	Product release	
	1/2 positive	Repeat the whole process New results:	
		0/2 positive	product release
		1/2 positive	Low contamination
		2/2 positive	Contamination
	2/2 positive	Contamination	

In case you want to identify a positive result, please send your PCR product to Minerva Biolabs GmbH. The PCR product will be purified by Minerva Biolabs. Sequencing will be done by an external sequencing service. The interpretation of your sequencing results will be supplied by Minerva Biolabs afterwards.
Attention: in case of a light or multiple contamination, the sequencing analysis might lead to wrong identification.

11. 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 successfully tested with the Microsart® RESEARCH Bacteria kit:

Mx3005P®, CFX96 Touch™, CFX96 Touch Deep Well™, LightCycler® 480 II, ABI, Rotor-gene, LightCycler I and II

Programming and Data Recording of Different qPCR Devices

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	40		
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

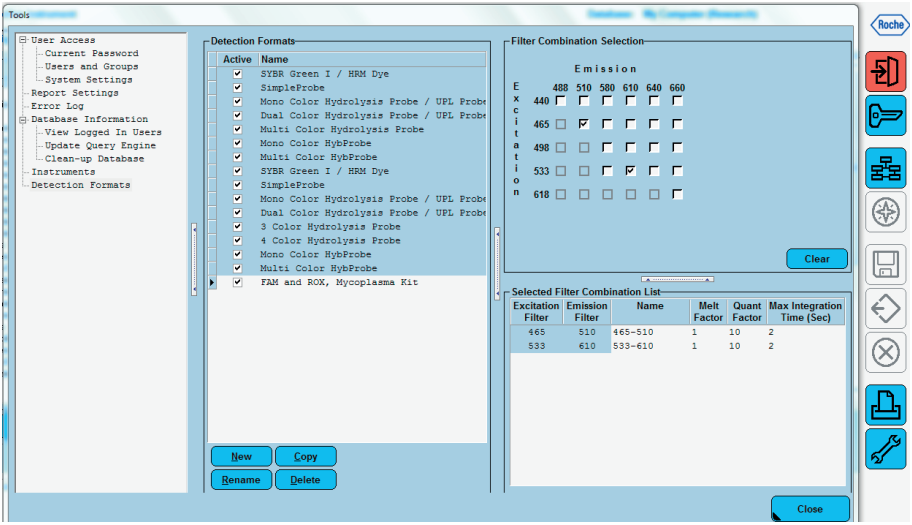
Analysis:
– Select the fluorescence channels Channel 1 (520 nm) and 3 (610 nm)
– Click on Quantification to generate the amplification plots and the specific Ct-values
– The threshold will be generated automatically
- Adapt the threshold line to 10 % of the maximum fluorescence level of the positive control (in case of duplicate determination, take the average of the maximum fluorescence levels)

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 "Bacteria Kit"
- Select the right filter combination by clicking the checkboxes with an 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	40		
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
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
Step Delay [Cycles]	0
Acquisition Mode	None

Data Analysis

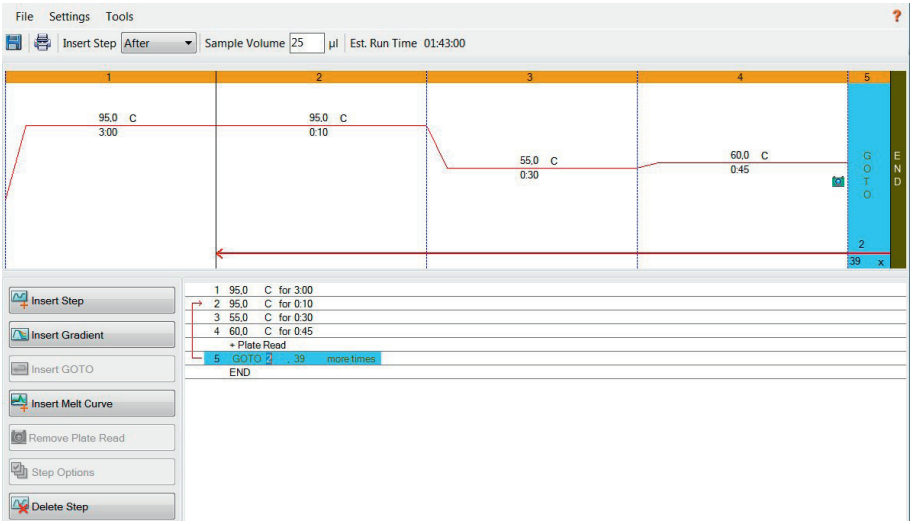
- Adapt the threshold line to 10 % of the maximum fluorescence level of the positive control (in case of duplicate determination, take the average of the maximum fluorescence levels)
- Select the Results tab to view specific Ct values

Bio-Rad CFX96 Touch™ / CFX96 Touch™ Deep Well

Run Setup Protocol Tab:

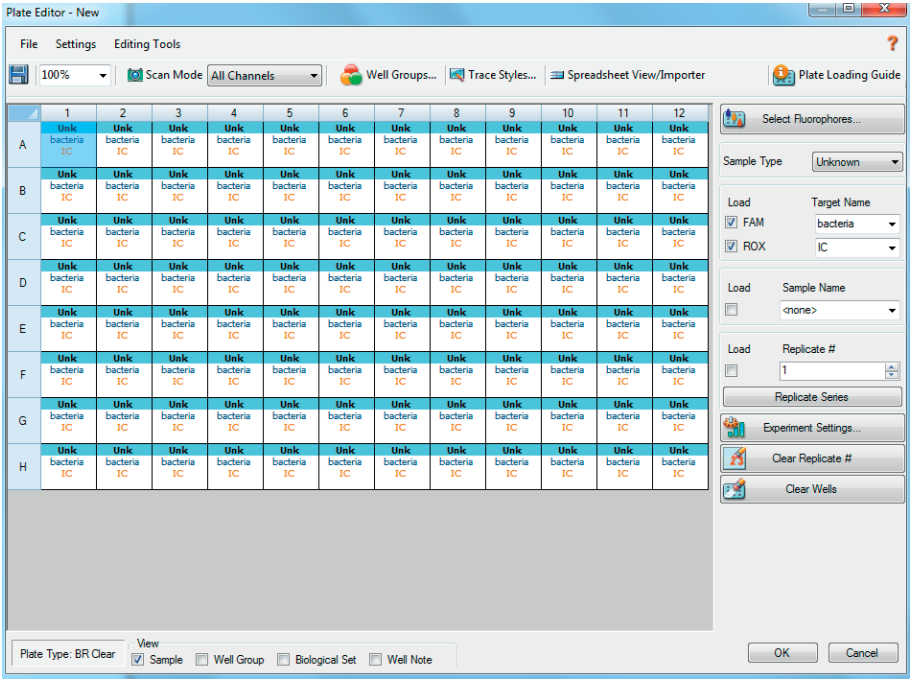
- Click File --> New --> Protocol to open the Protocol Editor and create a new protocol
- Select any step in either the graphical or text display
- Click the temperature or well time to directly edit the value

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
		GOTO Step 2, 39 more cycles		



Run Setup Plate Tab:

- Click File --> New --> Plate to open the Plate Editor and 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 bacteria 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 bacteria 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
- Remark: Amplification curves for which the baseline is not correctly calculated by the software, can be manually adapted
- By right-click 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
- Adapt the threshold line to 10 % of the maximum fluorescence level of the positive control (in case of duplicate determination, take the average of the maximum fluorescence levels)
- Evaluate the Ct-values according to chapter 10

RotorGene® 6000 (5-plex)

For the use of RotorGene® 6000, 0,1ml PCR tubes from Qiagen are recommended (Prod. No. 981106). Those tubes shall imperatively be used with the 72 well rotor from RotorGene® 6000.

1. Check the correct settings for the filter combination:

Target	Bacteria	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	40
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

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 10 % of the maximum fluorescence level of the positive control (in case of duplicate determination, take the average of the maximum fluorescence levels)
- 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	Bacteria	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 min

Program Step 2: Amplification

Cycles	40
Setting	Cycle
Denaturing	95 °C for 30 sec
Annealing	55 °C for 30 sec
Extension	60 °C for 45 sec

Analysis:

- Enter the following basic settings at the right task bar:
  Data: Delta RN vs. Cycle
  Detector: FAM™ and 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 10 % of the maximum fluorescence level of the positive control (in case of duplicate determination take the average of the maximum fluorescence levels)
- 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: 40 cycles	30 sec	95 °C
	30 sec	55 °C
	45 sec	60 °C data collection end

- 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“. The Threshold will be generated automatically
- Adapt the threshold line to 10 % of the maximum fluorescence level of the positive control (in case of duplicate determination take the average of the maximum fluorescence levels)
- Read the Ct-values in “Text Report“
- Evaluate the Ct-values according to chapter 10

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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Last technical revision: 2018-07-10

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
SMB95-1007	Microsart® ATMP Bacteria Patient	10 patients
SMB95-1008	Microsart® ATMP Bacteria	100 tests

Microsart® Calibration Reagent, 1 vial, 10^8 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
SMB95-2030	Bacillus subtilis
SMB95-2031	Pseudomonas aeruginosa
SMB95-2032	Kocuria rhizophila
SMB95-2033	Clostridium sporogenes
SMB95-2034	Bacteroides vulgatus
SMB95-2035	Staphylococcus aureus
SMB95-2036	Mycoplasma salivarium

Microsart® Validation Standard, 3 vials each, 10 CFU/vial for Mollicutes (SMB95-2011 - SMB95-2020) and 99 CFU/vial for other bacterial species (SMB95-2005-2010)

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
SMB95-2020	Mycoplasma salivarium
SMB95-2005	Bacillus subtilis
SMB95-2006	Pseudomonas aeruginosa

SMB95-2007	Kocuria rhizophila	
SMB95-2008	Clostridium sporogenes	
SMB95-2009	Bacteroides vulgatus	
SMB95-2010	Staphylococcus aureus	

DNA Extraction Kits

SMB95-2001	Microsart® Bacteria Extraction	50 extractions
SMB95-2003	Microsart® AMP Extraction (only for Mycoplasma qPCR)	50 extractions

PCR Clean™ (formerly DNA Remover™) *

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

PCR Clean™ Wipes*

15-2001	DNA Decontamination Wipes	120 wipes
15-2002	DNA Decontamination Wipes, refill sachets	5 x 120 wipes

Mycoplasma Off™ *

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

Mycoplasma Off™ Wipes*

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

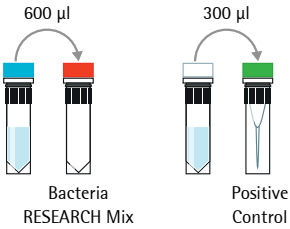
*** Distributed by Minerva Biolabs**

Procedure-Overview

Microsart® RESEARCH Bacteria

1. Rehydration of Reagents

Bacteria RESEARCH Mix and Positive Control

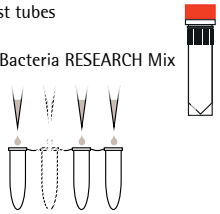


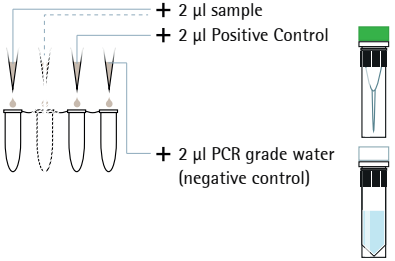
for 5 min RT
briefly
for 5 sec

2. Preparation of PCR Reactions

loading the test tubes

+ 23 µl Bacteria RESEARCH Mix





3. Starting the PCR Reaction

Rehydration Buffer
Bacteria RESEARCH Mix
PCR grade water
Positive Control

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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Order No.: 85037-559-37
Ver.07 | 2018

sartorius

Instructions for Use

Microsart® Validation Standard

For the validation of bacteria species (excluding mycoplasma)

Prod. No. SMB95-2005 Bacillus subtilis
Prod. No. SMB95-2006 Pseudomonas aeruginosa
Prod. No. SMB95-2007 Kocuria rhizophila
Prod. No. SMB95-2008 Clostridium sporogenes
Prod. No. SMB95-2009 Bacteroides vulgatus
Prod. No. SMB95-2010 Staphylococcus aureus

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
	Content
	Manufacturer

Contents

1. Intended Use

Microsart® Validation Standard is used for validating robustness and sensitivity of NAT-based detection methods in combination with cell cultures, cell culture media components and cell culture derived biologicals like cell autologous transplants and ATMPs.

2. Explanation of the Test

Microsart® Validation Standard requires to be validated with respect to sample matrix and lab precision. In addition, the method analysis for sterility testing shall show performance equal or better to the classical cultivation procedure. As most test and cell culture labs are frighten to cultivate bacteria, Microsart® ATMP Bacteria was validated as a method of choice for the sterility testing of cell culture samples including ATMPs. The direct comparison of Microsart® ATMP Bacteria with the classical culture method showed an equal performance between both methods.

Microsart® Validation Standards are not infectious and therefore safe in use. They are titrated to 99 CFU/ml, the sensitivity limit for sterility tests according to European Pharmacopoeia (EP) 2.6.1 Sterility. The bacteria strains used for the manufacture of Microsart® Validation Standard are low passage reference strains cultivated in suitable culture broth. The cultures are harvested in the early logarithmic phase of the growth to avoid an atypical high ratio of dead bacteria and plated on Agar medium for quantification based on Colony-forming Units (CFU).

3. Principle

Each vial contains 99 Colony-forming Units (CFU) of inactivated bacterial particles. The relevant sample matrix is added directly into the tube. The arising sample should be tested positive. The inactivated sample material cannot be used for the culture method. For highest sensitivity, the DNA should be extracted with a DNA-free kit. For this purpose we developed Microsart® Bacteria Extraction (SMB95-2001). The DNA extract can directly be used for PCR (we recommend to use Microsart® ATMP Bacteria SMB95-1008 or Microsart® RESEARCH Bacteria SMB95-1009).

4. Reagents

Each kit contains 6 vials of inactivated bacteria particles as well as 2 vials containing the same carrier matrix as the bacteria vials for the preparation of comparable negative controls. All samples are lyophilized for product stability reasons. All particles have been inactivated prior lyophilisation. The expiry date of the unopened package is specified on the package label. The kit components are stored until use at +2 to +8 °C and must be stored at ≤-18 °C after opening and rehydration.

Kit Component Label Information	Cat. No.	Quantity	Cap Color
Bacillus subtilis	SMB95-2005	6 x lyophilized	green
Pseudomonas aeruginosa	SMB95-2006		
Kocuria rhizophila	SMB95-2007		
Clostridium sporogenes	SMB95-2008		
Bacteroides vulgatus	SMB95-2009		
Staphylococcus aureus	SMB95-2010		
Negative Control		2 x lyophilized	white

5. Needed but not Included

Microsart® Validation Standard contains the positive and negative samples to perform the test. General industrial supplies and reagents, usually available in PCR laboratories are not included:

Consumables

- Laboratory gloves
- PCR Clean™ (Minerva Biolabs, Prod. No. 15-2025) and PCR Clean™ wipes (Minerva Biolabs, Prod. No. 15-2001)
- DNA-free pipette filter tips that must be free from bacterial DNA (Biosphere® filter tips from Sarstedt are recommended: 0.5-20 µl, Prod No. 70.1116.210; 2-100 µl, Prod No. 70.760.212; 20-300 µl, Prod. No. 70.765.210; 100-1000 µl. Prod. No. 70.762.211)

Equipment

- PCR cycler
- Isolator/glovebox (for PCR-setup)
- Vortex Mixer
- Heat block
- Microcentrifuge for 1.5 ml reaction tubes (Centrisart A-14, Prod. No. A-14-1EU)
- Pipettes
  mechanical
  0.5 – 10 µl Sartorius Prod. No. LH-729020
  10 – 100 µl Sartorius Prod. No. LH-729050
  100 – 1000 µl Sartorius Prod. No. LH-729070
  or electrical
  0,2 – 10 µl Sartorius Prod. No. 735021
  10 – 300 µl Sartorius Prod. No. 735061
  50 – 1000 µl Sartorius Prod No. 73508

- Rack for 1.5 ml tubes

For DNA extraction and PCR analysis, the following kits are required additionally:

- Bacterial DNA extraction system DNA-free.

Attention: Most DNA-extraction kits on the market are not DNA-free. For this reason, we recommend the Microsart® Bacteria Extraction kit (Sartorius Prod. No. SMB95-2001) intended for further Bacterial DNA amplification through qPCR.

- Bacterial DNA PCR detection system. We recommend the Microsart® ATMP Bacteria kit (Sartorius Prod. No. SMB95-1008), or the Microsart® RESEARCH Bacteria kit (Sartorius Prod. No. SMB951009)

6. Precautions

For in vitro use in research and quality control. This kit should be used only by trained persons. This kit does not contain hazardous substances and may be disposed of according to local regulations.

7. Procedure

1. Centrifuge the tubes 5 sec. with the “pulse” option or at 5000g to collect the lyophilized material at the bottom of the tube
2. Add 1 ml of your sample matrix to each vial
3. Incubate 5 min at room temperature
4. Vortex for 10 sec. and spin 5 sec. with the “pulse” option or at 5000g.
5. For DNA extraction use the recommended volume according to user instructions for DNA isolation

All samples must be equilibrated to room temperature prior use. It is highly recommended to perform suitable DNA extraction of the samples prior PCR application to reduce risk for inhibition and to maximize sensitivity. We recommend the DNA-free kit Microsart® Bacteria Extraction (SMB95-2001). Negative Control vials have been prepared just as the bacteria vials but do not contain any bacteria particles. The Negative Controls should be rehydrated with the same sample matrix and processed in parallel with a suitable number of replicates to validate the interpretation of the test results as correct positive.

8. Notes on the Procedure

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

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

3. Inhibition of PCR may be caused by the sample matrix added to the reagents. Negative controls should always be completed with the same sample matrix.

4. For each test setup, at least one negative control should be added that includes the sample preparation. Typical Ct-values for the analysis of this preparation using the Microsart® ATMP Bacteria kit are shown on the Certificate of Analysis.

5. Participation in external quality control programs, such as offered by Minerva Biolabs GmbH (www.minerva-biolabs.com) on a biannual base, is recommended

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 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.

Trademarks

Microsart is a registered trademark of Sartorius Stedim Biotech. Myocoplasma Off and PCR Clean are trademarks of Minerva Biolabs GmbH, Germany.

Last technical revision: 2018-06-18

9. 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
SMB95-1007	Microsart® ATMP Bacteria patient	10 patients
SMB95-1008	Microsart® ATMP Bacteria	100 tests
SMB95-1009	Microsart® RESEARCH Bacteria	25 tests

Microsart® Calibration Reagent, 1 vial, 10^8 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
SMB95-2030	Bacillus subtilis
SMB95-2031	Pseudomonas aeruginosa
SMB95-2032	Kocuria rhizophila
SMB95-2033	Clostridium sporogenes
SMB95-2034	Bacteroides vulgatus
SMB95-2035	Staphylococcus aureus
SMB95-2036	Mycoplasma salivarium

Microsart® Validation Standard, 3 vials each, 10 CFU/vial for Mollicutes (SMB95-2011 - SM95-2020) and 99 CFU/vial for other bacterial species (SMB95-2005 - SMB95-2010)

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
SMB95-2020	Mycoplasma salivarium

DNA Extraction Kit

SMB95-2001	Microsart® Bacteria Extraction	50 extractions
SMB95-2003	Microsart® AMP Extraction (only for Mycoplasma qPCR)	50 extractions

Vivaspin and Coating Buffer

SMB95-2002	Microsart® AMP Coating Buffer	20x 2 ml
VS 0641	Vivaspin 6 Polyethesulfone 100,000 MWCO	25 units
VS 0642	Vivaspin 6 Polyethesulfone 100,000 MWCO	100 units
VS 2041	Vivaspin 20 Polyethesulfone 100,000 MWCO	12 units
VS 2042	Vivaspin 20 Polyethesulfone 100,000 MWCO	48 units

UNG Carry over prevention*

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

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 Wipes	120 wipes
15-2002	DNA Decontamination Wipes, refill sachets	5× 120 wipes

Mycoplasma Off™ Wipes *

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

Mycoplasma Off™ Wipes *

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

*** Distributed by Minerva Biolabs**

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Application Note

December, 2018

Comparison of Microsart® ATMP Bacteria with the compendial culture method according to Ph. Eur. 2.6.1, JP 4.06 and USP <71>

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Keywords:
Real-time PCR, Microsart® ATMP Bacteria, Microsart® Bacteria Extraction, compendial sterility test, comparability study, sensitivity

Abstract

In this study the comparability of the Microsart® ATMP Bacteria Real-time PCR kit and the compendial sterility test was demonstrated in terms of the bacterial detection ability. Spiked samples, using 6 different bacterial species at concentration levels between 2.5 CFU/ml and 198 CFU/ml, were tested in parallel at an external contract lab according to Ph. Eur. 2.6.1, JP 4.06 and USP <71> [2] [3] [4]. These species included *Bacillus subtilis, Staphylococcus aureus, Clostridium sporogenes, Pseudomonas aeruginosa, Streptococcus pyogenes* and *Pseudomonas protegens*. A direct comparison with the culture method showed 100% of similarities between the results obtained through both methods.

The results of this study demonstrate that in addition to the classic sterility testing, rapid Real-time PCR-based detection of bacterial contamination contributes to a risk reduction and thus to patient safety.

Introduction

Sterility is a critical quality attribute of cellular therapeutics. Since microbial contamination of cell therapy products can potentially result in the deaths of the recipients, sterility testing is a critical component of the release testing for any cell therapy product. The current compendial sterility test takes 14 days before a contamination can be ruled out with certainty, which is too long for short shelf life cellular therapeutics and especially for autologous cell therapies intended to treat terminally ill patients. As a result there is an increasing demand for growth-independent rapid assays. Therefore a detection system consisting of the highly efficient DNA extraction protocol Microsart® Bacteria Extraction followed by the real-time PCR assay Microsart® ATMP Bacteria has been developed. A validation study was designed to evaluate the bacterial detection capability. The study was setup to meet requirements of the European Pharmacopeia chapter 5.1.6 [1]. Additionally in silico sequence alignment analysis demonstrated that Microsart® ATMP Bacteria is able to detect > 94 % of Gram positive and Gram negative bacteria. This feature greatly increases the chance to detect any kind of bacterial contamination, even including species difficult to detect with the classical culture method.

In this study comparability to the compendial culture method was demonstrated. Spiked samples were tested in parallel at an external contract lab according to Ph. Eur. 2.6.1, JP 4.06 and USP <71> [2] [3] [4].

Materials and Methods

EZ-CFU™ standards (quantified reference cultures; Microbiologics) of the six species listed in Table 1 were rehydrated in 2 ml of rehydration buffer according to the instructions for use [5]. The suspensions were diluted in DMEM + 5 % FBS to generate concentrations of 2x LOD_{95}, LOD_{95} and ½ LOD_{95}, and split into aliquots. LOD_{95} values of the six different bacterial species have been determined during PCR kit validation and are listed in Table 2.

A sample of each concentration was used for sterility testing (direct inoculation) at Labor LS. Each sample has been cultivated in thioglycolate medium and soya-bean casein medium for 14 days according to the recommendation of the guidelines. In parallel, aliquots were extracted in duplicates and analyzed using a CFX96 Real-time PCR instrument according to the instructions for use of the Microsart® Bacteria Extraction kit and the Microsart® ATMP Bacteria detection kit [6] [7]. The test setup is described in Table 3.

Species	Strain	Atmosphere
Bacillus subtilis	ATCC 6633	aerobic
Staphylococcus aureus	ATCC 6538	aerobic
Clostridium sporogenes	ATCC 19404	anaerobic
Pseudomonas aeruginosa	ATCC 9027	aerobic
Streptococcus pyogenes	ATCC 19615	aerobic
Pseudomonas protegens	ATCC 17386	aerobic

Table 1: Incubation conditions for each bacterial species

Species	LOD_{95}
Bacillus subtilis	25 CFU/ml
Staphylococcus aureus	25 CFU/ml
Clostridium sporogenes	50 CFU/ml
Pseudomonas aeruginosa	5 CFU/ml
Streptococcus pyogenes	99 CFU/ml
Pseudomonas protegens	10 CFU/ml

Table 2: LOD_{95} of the six bacterial species used in this study

Comparison with culture method with defined starting material quantity at external contract lab		
1	Spike DMEM + 5 % FBS with *Bacillus subtilis* at 2x LOD_{95}, LOD_{95} and ½ LOD_{95} One aliquot without spike is processed as NC.	
	1 ml of each sample is used for the sterility test (direct inoculation) at the external contract lab L+S AG	DNA is extracted from 1ml starting material with Microsart® Bacteria Extraction. Real-time PCR is performed according to Microsart® ATMP Bacteria.
2	Spike DMEM + 5 % FBS with *Clostridium sporogenes* at 2x LOD_{95}, LOD_{95} and ½ LOD_{95}. One aliquot without spike is processed as NC.	
	1 ml of each sample is used for the sterility test (direct inoculation) at the external contract lab L+S AG	DNA is extracted from 1ml starting material with Microsart® Bacteria Extraction. Real-time PCR is performed according to Microsart® ATMP Bacteria.
3	Spike DMEM + 5 % FBS with *Pseudomonas aeruginosa* at 2x LOD_{95}, LOD_{95} and ½ LOD_{95}. One aliquot without spike is processed as NC.	
	1 ml of each sample is used for the sterility test (direct inoculation) at the external contract lab L+S AG	DNA is extracted from 1ml starting material with Microsart® Bacteria Extraction. Real-time PCR is performed according to Microsart® ATMP Bacteria.
4	Spike DMEM + 5 % FBS with *Staphylococcus aureus* at 2x LOD_{95}, LOD_{95} and ½ LOD_{95}. One aliquot without spike is processed as NC.	
	1 ml of each sample is used for the sterility test (direct inoculation) at the external contract lab L+S AG	DNA is extracted from 1ml starting material with Microsart® Bacteria Extraction. Real-time PCR is performed according to Microsart® ATMP Bacteria.
5	Spike DMEM + 5 % FBS with *Streptococcus pyogenes* at 2x LOD_{95}, LOD_{95} and ½ LOD_{95}. One aliquot without spike is processed as NC.	
	1 ml of each sample is used for the sterility test (direct inoculation) at the external contract lab L+S AG	DNA is extracted from 1ml starting material with Microsart® Bacteria Extraction. Real-time PCR is performed according to Microsart® ATMP Bacteria.
6	Spike DMEM + 5 % FBS with *Pseudomonas protegens* at 2x LOD_{95}, LOD_{95} and ½ LOD_{95}. One aliquot without spike is processed as NC.	
	1 ml of each sample is used for the sterility test (direct inoculation) at the external contract lab L+S AG	DNA is extracted from 1ml starting material with Microsart® Bacteria Extraction. Real-time PCR is performed according to Microsart® ATMP Bacteria.

Table 3: Test setup for comparison between Microsart® ATMP Bacteria and compendial sterility test

Species	Microsart® ATMP Bacteria			Compendial culture method (External Lab)		
	2x LOD_{95}	LOD_{95}	½ LOD_{95}	2x LOD_{95}	LOD_{95}	½ LOD_{95}
Bacillus subtilis	33.16	34.23	35.47	Positive	Positive	Positive
	33.23	34.32	34.38			
Staphylococcus aureus	35.42	35.77	36.56	Positive	Positive	Positive
	34.13	35.67	39.90			
Clostridium sporogenes	34.20	34.87	35.45	Positive	Positive	Positive
	34.10	33.43	35.61			
Pseudomonas aeruginosa	36.40	36.74	37.22	Positive	Positive	**Negative**
	36.22	37.96	**No Cq**			
Streptococcus pyogenes	34.89	35.53	36.55	Positive	Positive	Positive
	35.09	35.93	35.88			
Pseudomonas protegens	34.14	34.38	36.52	Positive	Positive	Positive
	33.28	34.51	35.61			

Table 4: Results of Microsart® ATMP Bacteria and the compendial culture method according to Ph. Eur. 2.6.1, JP 4.06 and USP <71>

Discussion

In this study the equivalence of the Microsart® ATMP Bacteria detection kit and the compendial method was demonstrated in terms of the bacterial detection ability. Spiked samples were tested in parallel at an external contract lab according to Ph. Eur. 2.6.1, JP 4.06 and USP <71> [2] [3] [4]. A direct comparison with the culture method showed 100% of similarities between the results obtained through both methods. Only one single sample which contained 2.5 CFU/ml of *Pseudomonas aeruginosa* led to negative results for both methods. During validation of the Microsart® ATMP Bacteria kit an LOD_{95} of 5 CFU/ml was determined for *Pseudomonas aeruginosa* however, 2.5 CFU/ml are still detectable with a probability of 83%.

The results of this study conclusively demonstrate that in addition to the classic sterility testing, rapid Real-time PCR-based detection of bacterial contamination contribute to a risk reduction as they facilitate the availability of results prior treatment and therefore contribute to patient safety.

A respective assay to detect fungal contamination using the same technology and temperature profile as the bacteria assay will complete the portfolio in 2019 hence, enabling simultaneous results for total bacteria and fungi within the same PCR run in 3 hours instead of weeks. A rapid detection of such contaminants in short shelf life cellular therapeutics, especially autologous cell therapies is urgently needed prior administration to terminally ill patients.

References

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2. European Pharmacopoeia 8th edition, Strasbourg, FR; European Directorate for the Quality of Medicines, 2.6.1
3. Japanese Pharmacopoeia (JP): 4.06 Sterility Test, JP 16th edition, 2011, by the Ministry of Health, Labour and Welfare Ministerial Notification No. 65
4. United States Pharmacopeia (USP): <71> Sterility Tests, revision 2009Instructions for use: EZ-CFU™ Microorganisms, Microbiologics, 2014, Pl.046.ENG Rev B
5. Instructions for use: Microsart® Bacteria Extraction, Sartorius, Prod. No. SMB95-2001, Ver.06 | 2018
6. Instructions for use: Microsart® ATMP Bacteria, Sartorius, Prod. No. SMB95-1008, Ver.06 | 2018

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Application Note

January, 2019

Beta Test: Real-time PCR-based Presence/Absence Test for the Detection of Bacterial Contamination in ATMPs

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Keywords:
Real-time PCR, Microsart® ATMP Bacteria, Microsart® Bacteria Extraction, robustness, beta test participants, lab-to-lab precision, total bacteria, short shelf life products

Abstract

In this study the robustness of the Microsart® ATMP Bacteria real-time PCR kit was demonstrated by spiking DMEM + 5 % FBS with *Clostridium sporogenes* (ATCC 19404) at 2x LOD_{95}, LOD_{95} and ½ LOD_{95} and tested by five participants, representatives of the biopharmaceutical industry and hospital associated labs. The spiked samples were shipped frozen to all participants for running tests with the Microsart® Bacteria Extraction and Microsart® ATMP Bacteria at their site. *Clostridium sporogenes* has been selected for this study because this is one of the worst case organisms for the test system, showing highest limit of detection.

The results of this study demonstrate that rapid real-time PCR-based detection of bacterial contamination is a robust and suitable method which could contribute to a risk reduction and thus to patient safety.

Introduction

Sterility is a critical quality attribute of cellular therapeutics. Since microbial contamination of cell therapy products can potentially lead to life-threating infections of recipients sterility testing is a critical component of the release testing for any cell therapy product. The current compendial sterility test takes 14 days before a contamination can be ruled out with certainty which is too long for short shelf life cellular therapeutics, especially for autologous cell therapies intended to treat terminally ill patients. As a result there is an increasing demand for growth-independent rapid assays. Therefore, a detection system consisting of the highly efficient DNA extraction protocol of Microsart® Bacteria Extraction followed by the real-time PCR assay Microsart® ATMP Bacteria has been developed. A validation study was designed to evaluate the bacterial detection capability. The study was setup to meet requirements of the European Pharmacopeia chapter 5.1.6 [1]. Additionally in silico sequence alignment analysis demonstrated that Microsart® ATMP Bacteria is able to detect > 94 % of Gram positive and Gram negative bacteria. This feature enables the detection of any bacterial contamination, even including species difficult to detect with the classical culture method.

In this study the robustness of the Microsart® ATMP Bacteria real-time PCR kit with prior DNA extraction was demonstrated with the help of a beta test. Spiked samples were successfully tested by five participants.

Materials and Methods

Each of the beta test participants received the required kits and consumables (Table 1). The required lab instruments and equipment, i.e. real-time PCR cyclers, have been made available by the participants. Spiked samples (Table 2) that were unknown to the participants were provided by Sartorius.

Quantity	Product	Reactions
1	Microsart® ATMP Bacteria	100
1	Microsart® Bacteria Extraction	50
3	Biosphere® Filter Tips 1000 µl	-
2	Biosphere® Filter Tips 100 µl	-
2	Biopshere® Filter Tips 20 µl	-
2	PCR tubes á 12x 8-strip, High Profile	-
2	PCR tubes á 12x 8-strip, Low Profile	-
1	PCR Clean	-
1	PCR Clean Wipes	-
10	Pairs of Sleeve protectors	-
10	Hairnet	-
10	Mask	-

Table 1: Kits and consumables for the best test

Quantity	Volume per tube	Component	Label	Storage Temp.
1 tube	5 ml	Negative Extraction Control	NEC	2-8 °C
1 tube	15 ml	Matrix	Matrix	2-8 °C
8 tubes	1 ml	Unknown samples	1-8	-20 °C

Table 2: Unknown spiked samples for the beta test

All beta test participants followed the steps below:

1. 1 ml of the NEC was pipetted in each of 2 Processing Tubes (Processing Tubes are included in the Microsart® Bacteria Extraction kit) and labeled with "NEC 1" and "NEC 2".
2. 950 µl of the Matrix were pipetted in each of the tubes 1 to 8 (each of the tubes already contained 50 µl of unknown spiked sample) and mixed with a Vortex mixer.
3. The DNA of all 10 samples (2x NEC, 8x Unknown) were extracted following the protocol of the Microsart® Bacteria Extraction kit [2].
4. The PCR was setup following the protocol of the Microsart® ATMP Bacteria detection kit [3].
5. DNA extracts were analyzed in duplicates (Overview of sample number for DNA extraction and PCR setup is listed in Table 3).
6. For data analysis instructions in the manual were used.

	DNA Extraction Microsart® Bacteria Extraction	PCR Analysis Microsart® ATMP Bacteria
NEC	2	4
Unknown	8	16
PCR Negative Control (NTC)	n.a.	2
PCR Positive Control (PC)	n.a.	2
	Σ 10	Σ 24

Table 3: Overview of sample number for DNA extraction and PCR setup

Results

The expected results are listed in Table 4. The acceptance criterion was fulfilled if all samples containing *Clostridium sporogenes* (ATCC 19404) at concentrations of LOD_{95} and 2x LOD_{95} have been detected as positive, at least 6 out of 8 NECs of each participant were negative, 2 out of 2 negative PCR controls have to be negative and 2 out of 2 PCR positive controls were positive for each participant.

Sample Code and content	Expected result
NEC (Negative Extraction Control)	Negative
1 (1/2 LOD_{95} *C. sporogenes*)	Positive or negative
2 (2x LOD_{95} *C. sporogenes*)	Positive
3 (LOD_{95} *C. sporogenes*)	Positive
4 (No spike, Negative Extraction Control)	Negative
5 (LOD_{95} *C. sporogenes*)	Positive
6 (No spike, Negative Extraction Control)	Negative
7 (2x LOD_{95} *C. sporogenes*)	Positive
8 (1/2 LOD_{95} *C. sporogenes*)	Positive or negative
NTC (No Template Control)	Negative
PC (Positive Control)	Positive

LOD_{95} *C. sporogenes* = 99 CFU (*C. sporogenes* is worst case organism with the highest LOD_{95})

Table 4: Expected results of each beta test participant

Sample Name		Participant 1		Participant 2		Participant 3		Participant 4		Participant 5	
		FAM	ROX	FAM	ROX	FAM	ROX	FAM	ROX	FAM	ROX
NTC	PCR NTC 1	Negative	Correct	Negative	Correct	Negative	Correct	Negative	Correct	Negative	Correct
	PCR NTC 2	**Positive**	Correct	Negative	Correct	Negative	Correct	Negative	Correct	Negative	Correct
	PCR PC 1	Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct
	PCR PC 2	Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct
NEC	NEC 1	Negative	Correct	Negative	Correct	Negative	Correct	Negative	Correct	Negative	Correct
		Negative	Correct	**Positive**	Correct	Negative	Correct	Negative	Correct	Negative	Correct
	NEC2	Negative	Correct	Negative	Correct	Negative	Correct	Negative	Correct	Negative	Correct
		Negative	Correct	Negative	Correct	Negative	Correct	Negative	Correct	Negative	Correct
Unknown	1/2 LOD (Samples 1 and 8)	Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct
		Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct
		Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct
		Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct
	LOD (Samples 3 and 5)	Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct
		Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct
		Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct
		Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct
	2x LOD (Samples 2 and 7)	Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct
		Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct	Positive	Correct
		Positive	Correct	Positive	Correct	**Negative**	Correct	Positive	Correct	Positive	Correct
		Positive	Correct	Positive	Correct	**Negative**	Correct	Positive	Correct	Positive	Correct
	NEC (Samples 4 and 6)	Negative	Correct	Negative	Correct	Negative	Correct	Negative	Correct	Negative	Correct
		Negative	Correct	Negative	Correct	Negative	Correct	Negative	Correct	Negative	Correct
		Negative	Correct	Negative	Correct	Negative	Correct	Negative	Correct	Negative	Correct
		Negative	Correct	Negative	Correct	Negative	Correct	Negative	Correct	Negative	Correct

Table 3: Detail of the participants results

In this study all of the 1x LOD_{95} samples have been detected correctly. 4 out of 5 participants detected all 2x LOD_{95} samples positive. At least 6 NECs have been negative per participant. 1 participant detected a positive signal in one PCR NTC. All positive control reactions were positive.

Discussion

5 participants, representatives of the biopharmaceutical industry and hospital associated labs reported results. 4 out of 5 participants analyzed all the unknown samples correctly. Considering that one of the highest concentrated sample was not detected by one participant, while lower titer were, it can be considered that this was due to a handling error and not due to the sensitivity of the Microsart® ATMP Bacteria kit. Samples 4 and 6 were designed as unknown negative controls containing no DNA or cell spikes at all. All labs identified those samples correctly as negative.

At least 6 negative extraction controls have been negative per participant, therefore fulfilling the acceptance criterion. One participant detected a positive signal in one PCR No Template Control. We conclude that this is most likely due to a pipetting error as all other samples have been detected as expected by this participant.

Emphasizing that the participants have been untrained users, guided by Sartorius user instructions only, employing various different cycler instruments and mostly with no dedicated DNA-free lab conditions, this results demonstrate the lab-to-lab precision and robustness of the assay.

In summary, the Microsart® ATMP Bacteria real-time PCR kit with prior DNA extraction using specifically designed Microsart® Bacteria Extraction has been shown to be a rapid and robust tool for the detection of total bacterial contamination. Especially in time sensitive applications such as the quality control of short shelf life products like ATMPs, CAR-T cells and other cellular therapeutics it contributes to a risk reduction and thus to patient safety as it allows for QC test results prior patient treatment.

References

1. European Pharmacopoeia 8th edition, Strasbourg, FR; European Directorate for the Quality of Medicines, 5.1.6
2. Instructions for use: Microsart® Bacteria Extraction, Sartorius, Prod. No. SMB95-2001, Ver.06 | 2018
3. Instructions for use: Microsart® ATMP Bacteria, Sartorius, Prod. No. SMB95-1008, Ver.06 | 2018

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Microsart® ATMP Bacteria

Rapid detection of total bacteria in ATMPs prior treatment

User Benefits

- Detecting >95% of all known bacteria in one test
- 3h-result: prior to treatment
- Specific TaqMan® probes reduce false-positives
- Non-infectious validation standards
- Less pipetting: controls already included

Product Information

Contaminated ATMPs pose life-threatening risks for immunocompromised patients. Microbial release test results prior to treatment are critical to patient safety. Microsart® ATMP Bacteria enables the detection of bacterial contamination within 3 hours validated according to EP 5.1.6 and EP 2.6.27.

Introduction and Applications

Microsart® ATMP Bacteria utilizes quantitative real-time PCR (qPCR) as the method of choice for sensitive and reliable detection of bacterial contamination in Advanced Therapy Medicinal Products (ATMPs). The Microsart® ATMP Bacteria kit is validated according to EP 5.1.6 with respect to sensitivity, specificity and robustness for detection of bacteria in cell cultures, cell culture derived biologicals and ATMPs. The kit is not suitable to replace sterility testing according EP 2.6.1 or USP <71> yet. Microsart® ATMP Bacteria should be used as pre-check test to get rapid QC results for ATMPs prior patient treatment.

High Performance

Microsart® ATMP Bacteria detects >95 % of all known bacterial species. During kit validation sensitivity (5 to 99 CFU/ml) was proven for 18 bacterial species including 6 standard USP and EP strains. Comparability to the compendial method was demonstrated.

Fast Result

The Microsart® ATMP Bacteria kit is a fast and easy to use real-time PCR kit. The total procedure from DNA extraction to PCR result takes only about 3 hours.

TaqMan® Probes

The use of TaqMan® probes adds specificity to the PCR detection system with reduced risk of false-positives. No subsequent melting curve analysis is necessary as it is for SYBR-Green-based detection.

Summary

The Microsart® ATMP Bacteria kit is the optimal solution for all labs performing bacterial testing of cell cultures, cell culture derived biologicals and ATMPs. Microsart® ATMP Bacteria is the first validated bacterial detection kit which guarantees QC results prior to patient treatment.

Product Versions

a) Microsart® ATMP Bacteria Patient – contains all reagents for testing 10 patients including DNA extraction

b) Microsart® ATMP Bacteria (Bulk) – contains all reagents for 100 qPCR reactions without DNA extraction

Technical Data

Kit Components and Storage

Each kit contains color-coded tubes with ready-to-use Master Mix, buffer, positive control, internal control and PCR grade water. Microsart® ATMP Bacteria Patient additionally contains lysis buffer, suspension buffer and processing tubes.

The lyophilized kit components are stored at +2°C to +8°C and must be kept at < –18°C after rehydration or opening. The Master Mix should be constantly protected from light.

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

Kit Components

	Microsart® ATMP Bacteria (Bulk)	Microsart® ATMP Bacteria Patient	Cap color
ATMP Bacteria Mix	4 × lyophilized	10 × lyophilized	red
Rehydration Buffer	4 × 0.5 ml	10 × 0.5 ml	blue
Positive Control DNA	1× lyophilized	10 × lyophylized	green
Internal Control DNA	4 × lyophilized	10 × lyophilized	yellow
PCR grade Water	5 × 1.5 ml	20 × 1.5 ml	white
Lysis Buffer	–	10 × 1.8 ml	transparent
Suspension Buffer	–	10 × 1.5 ml	violet
Processing Tubes	–	10 × 3	–

Ordering Information

Description	Quantity	Order No.
Microsart® ATMP Bacteria (Bulk)	100 reactions	SMB95-1008
Microsart® ATMP Bacteria Patient	Reagents for 10 patients	SMB95-1007

Related Products

DNA Extraction kit		
Microsart® Bacteria Extraction	Reagents for 50 extractions	SMB95-2001
Detection Kits for qPCR		
Microsart® AMP Mycoplasma	25 reactions	SMB95-1001
Microsart® ATMP Mycoplasma	25 reactions	SMB95-1003
Microsart® RESEARCH Mycoplasma	25 reactions	SMB95-1005
Microsart® RESEARCH Bacteria	25 reactions	SMB95-1009

Microsart® Validation Standard according to EP 2.6.7 and USP <63> for Mycoplasma species and EP 2.6.1, EP 2.6.27 and USP <71> for other bacteria

3 vials with10 CFU/vial for Mycoplasma species and 6 vials with 99 CFU/vial for other bacteria

Order No.	Description
SMB95-2005	*Bacillus subtilis*
SMB95-2006	*Pseudomonas aeruginosa*
SMB95-2007	*Kocuria rhizophila*
SMB95-2008	*Clostridium sporogenes*
SMB95-2009	*Bacteroides vulgatus*
SMB95-2010	*Staphylococcus aureus*
SMB95-2011	*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*

Microsart® Calibration Reagent

1 vial, 108 genomes/vial

Order No.	Description
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*
SMB95-2030	*Bacillus subtilis*
SMB95-2031	*Pseudomonas aeruginosa*
SMB95-2032	*Kocuria rhizophila*
SMB95-2033	*Clostridium sporogenes*
SMB95-2034	*Bacteroides vulgatus*
SMB95-2035	*Staphylococcus aureus*
SMB95-2036	*Mycoplasma salivarium*

User-supplied Equipment and Material

Consumables

- Laboratory gloves
- PCR Clean™ (Minerva Biolabs, Prod. No. 15-2025) and PCR Clean™ wipes (Minerva Biolabs, Prod. No. 15-2001)
- DNA-free pipette filter tips that must be free from bacterial DNA (Biosphere® filter tips from Sarstedt are recommended: 0.5-20 µl, Prod No. 70.1116.210; 2-100 µl, Prod No. 70.760.212; 20-300 µl, Prod. No. 70.765.210; 100-1000 µl. Prod.No. 70.762.211)
- DNA-free PCR reaction tubes (PCR 8-SoftStrips with attached caps from Biozym are recommended: 0.1 ml Low Profile, Prod. No. 710975 and 0.2 ml High Profile, Prod. No. 710970)

Equipment

- DNA-free PCR reaction tubes (PCR 8-SoftStrips with attached caps from Biozym are recommended: 0.1 ml Low Profile, Prod. No. 710975 and 0.2 ml High Profile, Prod. No. 710970)
- Isolator/glovebox (further information, supplier and prices are available on request, please contact PCR@sartorius.com)
- Heat block
- Microcentrifuge for 1.5 ml reaction tubes (Centrisart A-14, Prod. No. A-14-1EU)
- Vortex Mixer
- qPCR device with filter sets for the detection of the fluorescence dyes FAM™ and ROX™ and suitable for 25 µl PCR reaction volumes
- Minicentrifuge for PCR-tubes
- Pipettes (Sartorius)
  mechanical:
  0.5 – 10 µl Sartorius Prod. No. LH-729020
  10 – 100 µl Sartorius Prod. No. LH-729050
  100 – 1000 µl Sartorius Prod. No. LH-729070
  or electrical:
  0.2 – 10 µl Sartorius Prod. No. 735021

For PCR support and recommendation please contact PCR@Sartorius.com.

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