Microsart® ATMP Sterile Release

Bacteria, fungi, and yeasts DNA extraction and qPCR detection kit
Prod. No. SMB95-1007

Reagents for 10 samples
For use in research and quality control

Manufactured by:
Minerva Biolabs GmbH | Schkopauer Ring 13 | 12681 Berlin | Germany
Symbols

**LOT** Lot No.

**REF** Order No.

**Expiration date**

**Store at**

**Σ** Contains reagents for 25 or 100 reactions

**Manufacturer**
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1. Intended Use

Microsart® ATMP Sterile Release kit is designed for DNA extraction of bacteria, fungi, and yeasts in cell culture derived biologicals, like Advanced Therapy Medicinal Products (ATMPs), and for direct detection based on real-time PCR (qPCR). Be aware that this product is not intended to be used as a diagnostic kit.

2. Explanation of the Test

Microsart® ATMP Sterile Release utilizes qPCR as the method of choice for sensitive and robust detection of bacterial or fungal contamination. To achieve highest sensitivity and avoid inhibitory effects in PCR testing, the DNA is extracted prior to PCR. Microsart® ATMP Sterile Release introduces a unique DNA extraction method, which reduces the risk of DNA contaminations, facilitating the detection of contaminants in cell culture and ATMPs via PCR. The subsequent qPCR assay can be performed with virtually any type of real-time PCR cycler able to detect the fluorescent 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 living material as all intact particles (e.g. live, dormant, non-culturable etc.) are detected.

3. Test Principle

Microsart® ATMP Sterile Release kit was optimized for the extraction and detection of genomic bacterial and fungal DNA in cell culture samples. The contamination risk has been minimized due to the reduced number of 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 while fungi and yeasts are specifically detected by amplifying a fragment of the highly conserved 18S rRNA coding region. Each 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 and fungal species. Both master mixes contain the polymerase. False negative results due to PCR inhibitors or improper DNA extraction are detected by using 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 Sterile Release. 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 by trained staff, only. A clean lab coat and disposable gloves should be worn 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 work with living strains, the local regulation for S2 laboratories must be followed.

6. This detection kit has been developed for 1ml starting volume. When using less than 1ml, please make sure that 99 cfu can be detected in the selected volume.

7. This kit is not validated for the extraction of mycoplasma DNA.

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. Positive controls 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. Controls should be handled in the same manner as the samples.

11. Assay inhibition may be caused by the sample matrix but also by sample elution buffers of incompatible or non-validated DNA extraction methods. Do not use reagents from another kit than the Microsart® ATMP Sterile Release.

12. Participation in external quality control programs, such as those offered by Minerva Biolabs GmbH (www.minerva-biolabs.com), is recommended.
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 thoroughly cleaned with PCR Clean™ (Minerva Biolabs, Prod. No. 15-2025) or PCR Clean™ Wipes (Minerva Biolabs, Prod. No. 15-2001) before and during the work process.
3. All materials that are introduced in the isolator/glovebox should be cleaned thoroughly with PCR Clean™. Do not forget to clean the airlock with PCR Clean™. Pipettes and gloves should be thoroughly cleaned 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 samples. It consists of 10 individual sample tests containing material for three DNA extractions, five bacteria PCR reactions, and five fungi PCR reactions. The expiry date of the unopened package is marked on the package label. The kit components are stored at +2 – +8 °C until use. Protect the SR Mixes from light.

<table>
<thead>
<tr>
<th>Kit Component Label Information</th>
<th>10 samples Order No. SMB95-1007</th>
<th>Cap Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis Buffer</td>
<td>10 x 1.8 ml</td>
<td>transparent</td>
</tr>
<tr>
<td>Suspension Buffer</td>
<td>10 x 0.4 ml</td>
<td>violet</td>
</tr>
<tr>
<td>Processing Tubes</td>
<td>10 x 3</td>
<td>-</td>
</tr>
<tr>
<td>Bacteria SR Mix</td>
<td>10 x lyophilized</td>
<td>red</td>
</tr>
<tr>
<td>Fungi SR Mix</td>
<td>10 x lyophilized</td>
<td>orange</td>
</tr>
<tr>
<td>Rehydration Buffer</td>
<td>10 x 0.3 ml</td>
<td>blue</td>
</tr>
<tr>
<td>Positive Control DNA</td>
<td>10 x lyophilized</td>
<td>green</td>
</tr>
<tr>
<td>Internal Control DNA</td>
<td>10 x lyophilized</td>
<td>yellow</td>
</tr>
<tr>
<td>PCR grade Water</td>
<td>20 x 0.3 ml</td>
<td>white</td>
</tr>
</tbody>
</table>

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 Sterile Release kit contains reagents for DNA extraction and 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 and fungal 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: 0.1ml Low Profile, Prod. No. 710 975 and 0.2 ml High Profile, Prod. No. 710 970)

Equipment
- Isolator/glovebox (further information, supplier and prices are available on request, please contact PCR@sartorius.com)
- Heat block with optional shaking function
- Microcentrifuge for 1.5 ml reaction tubes (Centrisart A-14, Prod. No. A-14-ΕΕU)
- Vortex
- qPCR device with filter sets for the detection of the fluorescent dyes FAM™ and ROX™ and suitable for 25 μl PCR reaction volumes
- Micocentrifuge for PCR tubes
- Pipettes (Sartorius)
  mechanical
  0.5 – 10 μl Sartorius Prod. No. LH-7290 20
  10 – 100 μl Sartorius Prod. No. LH-7290 50
  100 – 1000 μl Sartorius Prod. No. LH-7290 70
  or electrical
  0.2 – 10 μl Sartorius Prod. No. 7350 21
  10 – 300 μl Sartorius Prod. No. 7350 61
  50 – 1000 μl Sartorius Prod. No. 7350 81
- Rack for 15 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 the PCR tubes after Step 3 directly from Isolator 2 into Isolator 1. Please note that in this case you would need an additional airlock for Isolator 2.
7. Test Procedure

7.1 Recommendation for product release testing

Each sample should be extracted in duplicate along with a negative extraction control (NEC) (=3 extractions for 1 sample/product). Each obtained extract should be loaded 1 x for the bacteria and 1 x for the fungi qPCR assay. Additionally, each PCR assay should include 1 x PCR negative control (no-template control, NTC) and 1 x PCR positive control (PC).

<table>
<thead>
<tr>
<th>DNA extraction</th>
<th>PCR with Bacteria SR Mix</th>
<th>PCR with Fungi SR Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 x Sample</td>
<td>2 x Sample Extracts</td>
<td>2 x Sample Extracts</td>
</tr>
<tr>
<td>1 x NEC</td>
<td>1 x NEC</td>
<td>1 x NEC</td>
</tr>
<tr>
<td></td>
<td>1 x PC</td>
<td>1 x PC</td>
</tr>
<tr>
<td></td>
<td>1 x NTC</td>
<td>1 x NTC</td>
</tr>
<tr>
<td>Σ 3 extractions</td>
<td>Σ 5 PCR reactions</td>
<td>Σ 5 PCR reactions</td>
</tr>
</tbody>
</table>

7.2 Sample Collection and Storage

The kit has been validated using a maximum cell concentration of $10^6$ cells/ml. Notably, the assay can be performed with different types of cell culture-derived material. Therefore, the optimal sampling parameters, like volume or cell number, can vary according to the specific characteristics of the sample (e.g. medium, cell type) and may require optimization of the procedure.

1. max. 1 ml of cell culture or cell culture supernatant liquid material is transferred into a provided DNA-free 15 ml processing tube (transparent cap).

2. Spin down for 15 minutes at a speed of at least 16,200 x g to sediment particles. **Attention:** Make sure to position the tubes in the centrifuge in order to obtain a pellet on the back side of the tube, as described in the figure below.
3. Discard the supernatant carefully and completely as described in 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 obtain a 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 (optional with shaking) 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 in the figure in chapter 7.2.

5. Remove supernatant carefully and completely, following the explanations in chapter 7.2. 
   **Attention:** Make sure not to disturb or aspirate the pellet in the process. 
   **Attention:** There is a higher 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

<table>
<thead>
<tr>
<th>Step</th>
<th>Reagents</th>
<th>Cap Colors</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bacteria SR Mix, Fungi SR Mix, Internal Control DNA, Positive Control</td>
<td>red cap, orange cap, yellow cap, green cap</td>
<td>Centrifuge briefly</td>
</tr>
<tr>
<td>2</td>
<td>Bacteria SR Mix, Fungi SR Mix</td>
<td>red cap, orange cap</td>
<td>Add 90 µl Rehydration Buffer (blue cap)</td>
</tr>
<tr>
<td>3</td>
<td>Internal Control DNA</td>
<td>yellow cap</td>
<td>Add 100 µl PCR grade Water (white cap)</td>
</tr>
<tr>
<td>4</td>
<td>Positive Control DNA</td>
<td>green cap</td>
<td>Add 100 µl PCR grade Water (white cap)</td>
</tr>
<tr>
<td>5</td>
<td>Bacteria SR Mix, Fungi SR Mix, Internal Control DNA, Positive Control DNA</td>
<td>red cap, orange cap, yellow cap, green cap</td>
<td>Incubate 5 min at room temperature</td>
</tr>
<tr>
<td>6</td>
<td>Bacteria SR Mix, Fungi SR Mix, Internal Control DNA, Positive Control DNA</td>
<td>red cap, orange cap, yellow cap, green cap</td>
<td>Vortex briefly</td>
</tr>
</tbody>
</table>
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 has been loaded.

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 adding 6 µl of Internal Control DNA (yellow cap) to each rehydrated Mix (red cap for Bacteria SR Mix; orange cap for Fungi SR Mix).
2. Homogenize the reaction mix by tapping carefully against the tube. Spin briefly.

Attention:
If the Internal Control DNA was added to the sample during DNA extraction, add 15 µl of the Mix (red cap for Bacteria SR Mix; orange cap for Fungi SR Mix) directly to each PCR tube. In this case, do not forget to add 1µl of Internal Control DNA to NTC and PC.
7.6 Loading of 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 master mix 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 master mix setup or DNA extraction.

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

4. Close and spin all PCR tubes briefly, load the qPCR cycler and start the program.

7.7 Start of 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 fluorescent dyes FAM™ and ROX™.

3. **FAM™:** adapt the threshold line to 10 % of the maximum fluorescence level of the positive controls. **ROX™:** adapt the threshold line to 10 % of the maximum fluorescence level of the NTCs. In case of duplicate determination take the average of the maximum fluorescence levels. See chapter 10.

4. Analyze the calculation of the Ct values for negative controls, positive controls and samples.
8. Interpretation of Results

The presence of 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 Ct 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 correspondence of the internal control channel. Target 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 contaminant DNA loads in the sample.

8.1 Yes/No Evaluation

<table>
<thead>
<tr>
<th>Detection of contaminants:</th>
<th>Internal Control:</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAM™ channel</td>
<td>ROX™ channel</td>
<td></td>
</tr>
<tr>
<td>positive (Ct &lt; 40)</td>
<td>irrelevant</td>
<td>Target DNA positive</td>
</tr>
<tr>
<td>negative (no Ct)</td>
<td>negative**</td>
<td>if used as PCR control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>if used as process control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Extraction or/and PCR inhibition</td>
</tr>
<tr>
<td>negative (no Ct)</td>
<td>positive (Ct &lt; 40)</td>
<td>Target DNA negative</td>
</tr>
</tbody>
</table>

*PCR inhibition might be caused by the 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 negative samples (FAM™: no Ct) must show Ct values in the range of +/- 2 cycles (ROX™) of the PCR negative control (NTC). If used as process control, Internal Control of negative samples (FAM™: no Ct) must show Ct values in the range of +/- 3 cycles (ROX™) of the NTC.
8.2 Total Analysis and recommended actions for product release testing

<table>
<thead>
<tr>
<th>Sample</th>
<th>Result</th>
<th>Interpretation</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTC</td>
<td>NTC negative</td>
<td>Valid PCR</td>
<td>Interpret specimen results</td>
</tr>
<tr>
<td></td>
<td>NTC positive</td>
<td>PCR contamination</td>
<td>Repeat PCR only</td>
</tr>
<tr>
<td>PC</td>
<td>PC positive</td>
<td>Valid PCR</td>
<td>Interpret specimen results</td>
</tr>
<tr>
<td></td>
<td>PC negative</td>
<td>Failed PCR</td>
<td>Repeat PCR only</td>
</tr>
<tr>
<td>NEC</td>
<td>NEC negative</td>
<td>Valid PCR</td>
<td>Interpret specimen results</td>
</tr>
<tr>
<td></td>
<td>NEC positive</td>
<td>Contamination during extraction or PCR</td>
<td>Repeat the whole process incl. DNA extraction and PCR</td>
</tr>
<tr>
<td>Specimen</td>
<td>0/2 positive</td>
<td>No contamination</td>
<td>Release</td>
</tr>
<tr>
<td></td>
<td>1/2 positive</td>
<td>Possible contamination</td>
<td>Repeat the whole process* incl. DNA extraction, PCR, and analysis. If result is confirmed, no release.</td>
</tr>
<tr>
<td></td>
<td>2/2 positive</td>
<td>Contamination</td>
<td>No release</td>
</tr>
</tbody>
</table>

*If NTC is also positive, repeat PCR only.

Use these analytical guidelines for each set of obtained results, namely for bacteria or fungi contaminations, separately.

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 performed by an external sequencing service. The interpretation of your sequencing results will be supplied by Minerva Biolabs afterwards.

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

The protocol can be performed with any type of real-time PCR cycler able to detect the fluorescent dyes FAM™ and ROX™.

The following qPCR cyclers were used for the validation of Microsart® Sterile Release: QuantStudio™, Mx3005P™, CFX96 Touch™, CFX96 Touch Deep Well™, ABI Prism® 7500, Rotor-Gene® Q/Rotor-Gene®.

In addition, Microsart® ATMP Sterile Release kit was successfully tested with the Light-Cycler® 480 II. A detailed protocol is available on request.

QuantStudio™
- Click File -> New Experiment -> Experiment Setup, to open the setup menu.
  - Select “Properties” from the menu bar to open the “Experiment Properties” tab. In this tab, assign an experiment name (“Name”) and make sure that “Chemistry” is set to “TaqMan® Reagents” and that the “Run mode” option is set to “Standard”
- Select “Method” in the menu bar to open the “Experiment Method” tab.
- Adjust the reaction volume and the cover temperature by setting “Volume” to 25 µl and “Cover” to 105 °C.
- Program a “Hold Stage” of 3 min at 95 °C.
- In “PCR Stage”, perform “Step1” (denaturation step) at 95 °C for 30 sec, “Step2” (annealing step) at 55 °C for 30 sec, and “Step3” (elongation step) at 60 °C for 45 sec. Enable the data readout during the elongation step by clicking on the camera symbol. Set the number of cycles to 40. See also figure below for an overview of these settings.
- Select “Plate” in the menu bar to open the “Assign Targets and Samples” tab.
  - Important: Set the “Passive Reference” to “None”!!!!
- Click on the “Advanced Setup” tab to define your targets and samples.
- In the “Targets” section: for the assay target, choose FAM™ as a reporter and NFQ-MGB as a quencher. For the internal control target, choose ROX™ as a reporter and NFQ-MGB as a quencher.
- Enter your samples names in the “Samples” section.
- Select the wells containing your samples (from the graphical overview of the plate), enable the targets, and assign the appropriate sample name by clicking on the respective check boxes.
- Select “Run” from the menu bar and click on START RUN to start the PCR run.
Data Analysis
- To enable thresholds setting, please follow the specific instructions provided in the manual of your cycler.
- FAM™: adapt the threshold line to 10% of the maximum fluorescence level of the positive control. ROX™: adapt the threshold line to 10% of the maximum fluorescence level of the NTCs. 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 target DNA 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 or fungi 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.
- To enable thresholds setting, please follow the specific instructions provided in the manual of your cycler.
- FAM™: adapt the threshold line to 10% of the maximum fluorescence level of the positive control. ROX™: adapt the threshold line to 10% of the maximum fluorescence level of the NTCs. In case of duplicate determination take the average of the maximum fluorescence levels.
- Evaluate the Ct values according to chapter 9.
Rotor-Gene® 6000 (5-plex)

For the use of Rotor-Gene® 6000, 0.1 ml PCR tubes from Qiagen are recommended (Prod. No. 981106). Those tubes shall imperatively be used with the 72 well rotor from Rotor-Gene® 6000.

1. Check the correct settings for the filter combination:

<table>
<thead>
<tr>
<th>Target</th>
<th>Bacteria or Fungi/Yeasts</th>
<th>Internal Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filter</td>
<td>green</td>
<td>orange</td>
</tr>
<tr>
<td>Wavelength</td>
<td>470—510 nm</td>
<td>585-610 nm</td>
</tr>
</tbody>
</table>

2. Program the Cycler:

Program 1: Pre-incubation

<table>
<thead>
<tr>
<th>Setting</th>
<th>Hold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold Temperature</td>
<td>95 °C</td>
</tr>
<tr>
<td>Hold Time</td>
<td>3 min 0 sec</td>
</tr>
</tbody>
</table>

Program Step 2: Amplification

<table>
<thead>
<tr>
<th>Setting</th>
<th>Cycling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycles</td>
<td>40</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C for 30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>55 °C for 30 sec</td>
</tr>
<tr>
<td>Elongation</td>
<td>60 °C for 45 sec → acquiring to Cycling A (green and orange)</td>
</tr>
<tr>
<td>Gain setting</td>
<td>automatic (Auto-Gain)</td>
</tr>
<tr>
<td>Slope Correct</td>
<td>activated</td>
</tr>
<tr>
<td>Ignore First</td>
<td>deactivated</td>
</tr>
</tbody>
</table>
Analysis:

Please analyze the results of the bacteria and fungi assays, separately.
- Open the menu Analysis
- Select Quantitation
- Check the required filter set (green and orange) according to the table above 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 included in the run and auto threshold was selected):
  - In window “CT Calculation” set the threshold value to 0-1
  - Pull the threshold line into the graph. For FAM ™: adapt the threshold line to 10 % of the maximum fluorescence level of the positive controls. For ROX ™: adapt the threshold line to 10 % of the maximum fluorescence level of the NTCs. 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:

<table>
<thead>
<tr>
<th>Target</th>
<th>Bacteria or Fungi/Yeasts</th>
<th>Internal Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filter</td>
<td>FAM™</td>
<td>ROX™</td>
</tr>
<tr>
<td>Wavelength</td>
<td>470 - 510 nm</td>
<td>585 - 610 nm</td>
</tr>
<tr>
<td>Quencher</td>
<td>none</td>
<td>none</td>
</tr>
</tbody>
</table>

**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**

<table>
<thead>
<tr>
<th>Setting</th>
<th>Hold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>95 °C</td>
</tr>
<tr>
<td>Incubation time</td>
<td>3 min</td>
</tr>
</tbody>
</table>

**Program Step 2: Amplification**

<table>
<thead>
<tr>
<th>Cycles</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Setting</td>
<td>Cycle</td>
</tr>
<tr>
<td>Denaturing</td>
<td>95 °C for 30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>55 °C for 30 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>60 °C for 45 sec</td>
</tr>
</tbody>
</table>
Analysis:

- Please analyze the results of the bacteria and fungi assays, separately.
- 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 “Analyze” within the report window.
- Pull the threshold line into the graph. For FAM™: adapt the threshold line to 10 % of the maximum fluorescence level of the positive controls. For ROX™: adapt the threshold line to 10 % of the maximum fluorescence level of the NTCs. In case of duplicate determination take the average of the maximum fluorescence levels.
- Samples showing no Ct value can be considered as negative.
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“:

<table>
<thead>
<tr>
<th>Segment 1</th>
<th>cycle 3 min</th>
<th>95 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Segment 2:</td>
<td>40 cycles</td>
<td>30 sec 95 °C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 sec 55 °C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>45 sec 60 °C data collection end</td>
</tr>
</tbody>
</table>

at menu “Run Status“ select ”Run“ and start the cycler by pushing „Start“

Analysis of raw data:
Please analyze the results of the bacteria and fungi assays, separately.
In the window “Analysis” tab on ”Analysis Selection / Setup“ to analyze the marked positions.
Ensure that in window “algorithm enhancement“ all options are activated:
Amplification-based threshold
Adaptive baseline
Moving average
Click on “Results“ and “Amplification Plots“. The Threshold will be generated automatically.
FAM™: adapt the threshold line to 10 % of the maximum fluorescence level of the positive controls. ROX™: adapt the threshold line to 10 % of the maximum fluorescence level of the NTCs. 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 9.
10. Related Products

Detection Kits for qPCR

<table>
<thead>
<tr>
<th>Kit Code</th>
<th>Name</th>
<th>Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMB95-1001/1002</td>
<td>Microsart® AMP Mycoplasma</td>
<td>25/100 tests</td>
</tr>
<tr>
<td>SMB95-1003/1004</td>
<td>Microsart® ATMP Mycoplasma</td>
<td>25/100 tests</td>
</tr>
<tr>
<td>SMB95-1005/1006</td>
<td>Microsart® RESEARCH Mycoplasma</td>
<td>25/100 tests</td>
</tr>
<tr>
<td>SMB95-1009</td>
<td>Microsart® RESEARCH Bacteria</td>
<td>25 tests</td>
</tr>
<tr>
<td>SMB95-1008</td>
<td>Microsart® ATMP Bacteria</td>
<td>100 tests</td>
</tr>
<tr>
<td>SMB95-1012</td>
<td>Microsart® ATMP Fungi</td>
<td>100 tests</td>
</tr>
<tr>
<td>SMB95-1014/1013</td>
<td>Microsart® RESEARCH Fungi</td>
<td>25/100 tests</td>
</tr>
</tbody>
</table>

Microsart® Calibration Reagent, 1 vial, 10^6 genomes / vial (bacteria, including Mollicutes)

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

Microsart® Calibration Reagent, 1 vial, 10^6 genomes / vial (fungi)

<table>
<thead>
<tr>
<th>Kit Code</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMB95-2044</td>
<td>Candida albicans</td>
</tr>
<tr>
<td>SMB95-2045</td>
<td>Aspergillus brasiliensis</td>
</tr>
<tr>
<td>SMB95-2046</td>
<td>Aspergillus fumigatus</td>
</tr>
<tr>
<td>SMB95-2047</td>
<td>Penicillium chrysogenum</td>
</tr>
<tr>
<td>SMB95-2048</td>
<td>Candida glabrata</td>
</tr>
<tr>
<td>SMB95-2049</td>
<td>Candida krusei</td>
</tr>
<tr>
<td>SMB95-2050</td>
<td>Candida tropicalis</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Kit Code</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMB95-2011</td>
<td>Mycoplasma arginini</td>
</tr>
<tr>
<td>SMB95-2012</td>
<td>Mycoplasma orale</td>
</tr>
</tbody>
</table>
Mycoplasma gallisepticum
Mycoplasma pneumoniae
Mycoplasma synoviae
Mycoplasma fermentans
Mycoplasma hyorhinis
Acholeplasma laidlawii
Spiroplasma citri
Mycoplasma salivarium

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

Mycoplasma gallisepticum
Mycoplasma pneumoniae
Mycoplasma synoviae
Mycoplasma fermentans
Mycoplasma hyorhinis
Acholeplasma laidlawii
Spiroplasma citri
Mycoplasma salivarium

DNA Extraction Kit

Microsart® ATMP Extraction (for bacteria and fungi)
Microsart® AMP Extraction (for mycoplasma)
Proteinase K**

PCR Clean™ **

DNA Decontamination Reagent, spray bottle 250 ml
DNA Decontamination Reagent, refill bottles 4 x 500 ml

PCR Clean™ Wipes**

DNA Decontamination Reagent, Wipes 50 wipes
DNA Decontamination Reagent, refill sachets 5 x 50 wipes

** Distributed by Minerva Biolabs
Short Instructions

1. Sample Collection

- 1 ml Sample Material or Negative Extraction Control (NEC)
- + 500 µl Lysis Buffer (transparent cap)
- 15 min, ≥ 16,200 x g
- Discard supernatant
- Store at ≤ -18 °C

Optional:
- Add Internal Control from Microsart® ATMP Sterile Release

2. DNA Extraction

- 1 ml Sample Material or Negative Extraction Control (NEC)
- Processing tubes
- + 500 µl Lysis Buffer (transparent cap)
- ≥ 30 sec vigorously
- 80 °C, 10 min
- ≥ 16,200 x g, 10 min
- Remove supernatant carefully

- + 100 µl Suspension Buffer (violet cap)
- ≥ 30 sec vigorously
- DNA ready for PCR

3. Rehydration of Reagents

- 90 µl Bacteria SR Mix
- 90 µl Fungi SR Mix
- 100 µl Positive Control DNA
- 100 µl Internal Control DNA
- Incubate briefly for 5 min RT
- vortex for 5 sec

This procedure overview is not a substitute for the detailed manual.
4. Preparation of PCR Reaction Mix

a) Internal Control added during DNA extraction

- 15 µl Bacteria SR Mix (red cap)
- or
- Fungi SR Mix (orange cap)

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

b) Internal Control not added during DNA extraction

- 6 µl

5. Addition of Samples and Controls

- + 10 µl NTC: PCR grade water (white cap)
- + 10 µl NEC
- + 10 µl Sample Extract 1
- + 10 µl Sample Extract 2
- + 10 µl PC (green cap)

Close lid tightly ∅ briefly

6. Start of the qPCR Reaction

Start PCR program

- 95°C 3 min
- 95°C 30 sec
- 55°C 45 cycles
- 60°C 30 sec
- 95°C 30 sec

Rehydration Buffer | incubate | storage +2 - +8 ºC after rehydration ≤ -18 ºC
Mycoplasma Mix | vortex | PCR grade Water | centrifuge | Positive Control | add | Internal Control

This procedure overview is not a substitute for the detailed manual. ST_SI_Microsart®-ATMP-Sterile Release_03_EN
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