SepsiTest™

Pathogen DNA Extraction and PCR Analysis

Direct universal PCR detection of
bacteria and fungi in whole blood (1ml)

Positive PCR control DNA and sequencing
primers included

IVD CE

– For *in-vitro* diagnostic use –

Molzym

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Version 3.0

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Contents page

Kit Information

Kit Contents – *SepsiTest™*

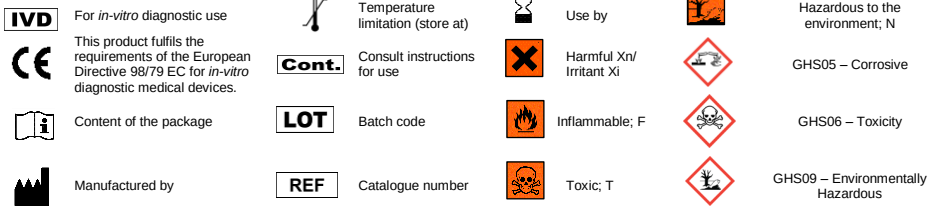
DNA Isolation	**12 tests**	**24 tests**
Kit 1 - Buffers & Consumables (store at +18 to +25°C), in DNA-free bags		
A) Extraction Buffers, in DNA-free bags		
CM	6x 1.0ml	12x 1.0ml
DB1	6x 1.0ml	12x 1.0ml
RS	6x [4x 1.0ml]	12x [4x 1.0ml]
RL	6x 0.32ml	12x 0.32ml
RP	6x 0.6ml	12x 0.6ml
CS	6x 1.0ml	12x 1.0ml
AB	6x 1.0ml	12x 1.0ml
WB	6x 1.6ml	12x 1.6ml
WS	6x 1.6ml	12x 1.6ml
ES	6x 0.4ml	12x 0.4ml
B) Consumables, in DNA-free bags		
ST - Sample tubes, 2.0ml	6x 4	12x 4
C) Consumables, in DNA-free bags		
SC – Spin columns in 2.0ml *Collection tubes*	6x 4	12x 4
CT - Collection tubes, 2.0ml	6x 8	12x 8
ET – Elution tubes, 1.5ml	6x 4	12x 4
Kit 2 – Enzymes & Reagents (store at -15 to -25°C), in white boxes		
MolDNase B, solution	2x [3x 0.04ml]	4x [3x 0.04ml]
BugLysis, solution	2x [3x 0.08ml]	4x [3x 0.08ml]
ß-mercaptoethanol, solution	2x [3x 0.08ml]	4x [3x 0.08ml]
Proteinase K, solution	2x [3x 0.08ml]	4x [3x 0.08ml]

Continued on next page

PCR Detection and Identification	12 tests	24 tests
Kit 3 - PCR Reagents (store at -15 to -25°C), in white boxes		
MA Bac, Mastermix Assay Bacteria, 2.5x conc.	2x 0.30ml	2x [2x 0.30ml]
MA Yeasts, Mastermix Assay Yeasts, 2.5x conc.	2x 0.30ml	2x [2x 0.30ml]
MolTaq 16S	2x 0.05ml	2x [2x 0.05ml]
H_2O - DNA-free PCR-grade water	2x 0.75ml	2x [2x 0.75ml]
DS - DNA staining solution, 10x conc.	1x 0.30ml	2x 0.30ml
Kit 4 - PCR Controls & Detection Reagents (store at -15 to -25°C)		
A) PCR and Detection Reagents (white box)		
MA IC - Mastermix Assay Internal Control, 2.5x conc.	1x 0.42ml	2x 0.42ml
MolTaq 16S	1x 0.05ml	2x 0.05ml
H_2O - DNA-free PCR-grade water	1x 0.75ml	1x 0.75ml
DS - DNA staining solution, 10x conc.	1x 0.3ml	1x 0.3ml
LS - Gel loading solution, 6x conc.	1x 0.4ml	1x 0.4ml
SM - DNA size marker	1x 0.24ml	1x 0.24ml
SeqGP16 - Sequencing primer (bacteria)	1x 0.1ml	2x 0.1ml
SeqGN16 - Sequencing primer (bacteria)	1x 0.1ml	2x 0.1ml
SeqYeast18 - Sequencing primer (fungi)	1x 0.1ml	2x 0.1ml
B) Positive PCR Control (bag)		
DNA Standard P1, for PCR positive control runs	1x 0.3ml	1x 0.3ml
DNA dilution buffer (for P1)	1x 25ml	2x 25ml
Consumables PCR Detection and Identification (store at +18 to +25°C), in Kit 1		
MT - Mastermix tubes, 1.5ml for Kit 3	1x 50	2x 50
MT - Mastermix tubes, 1.5ml for Kit 4A	1x 50	1x 50
Manuals (in Kit 1)	**12 tests**	**24 tests**
Manual	1x	1x
Short manual	2x	2x

Symbols

Symbols used in labelling and in section 'Risk and Safety Phrases' (pages 8 to 9).



Storage and Stability

Guarantee for ***full performance*** of reagents and buffers is given for ***12 months*** at the conditions specified and is guaranteed only if ***packed material*** is ***undamaged*** upon arrival. Once opened, the vials have to be used as specified by the protocol.

Guarantee for full performance of ***SepsiTest™*** as specified in this manual is only valid if storage conditions are followed. Please take care that the vials of the DNA Isolation unit, Kit 2 (Enzymes and Reagents), and the PCR Detection unit, including Kit 3 (PCR Reagents) and Kit 4A (PCR Controls and Detection Reagents), have to be stored at -15 to -25°C upon delivery.
It is important to note that the DNA staining solution (***DS***, Kits 3 and 4A) is sensitive to light and should be stored in the dark during handling and storage. ***Do not freeze again*** and store at +4 to +12°C for further use.
The reagents of Kit 4B (Positive PCR Control) should be stored at +4 to +12°C after the first handling.
Buffers and consumables of the DNA Isolation unit (Kit 1, bags A to C) should be stored dry in the dark and at room temperature (+18 to +25°C). Opened bags of vials/ consumables can be stored at room temperature (+18 to +25°C) for 4 days.

The DNA Isolation Unit, Kits 1 and 2, is provided in vials containing reagents to perform 4 sample extractions to minimise the risk of carry-over contamination.

Product Use Limitations

SepsiTest™ is intended as a kit for ***in-vitro*** **diagnostic use with whole blood samples**. ***SepsiTest™*** is not claimed or intended to be used for the detection and identification of any specific pathogen or not for clinical use of other specimens than specified above, including diagnostic, therapeutic or blood banking. The CE marking is limited to the detection of bacteria and fungi without further taxonomic differentiation at the detection level. ***SepsiTest™*** is not intended to be used as the only diagnostic tool for the presence of bacteria and fungi in the specimens, but rather as a means of rapid detection of pathogens flanking standard culturing analysis. It is emphasised that, provided all controls are as expected, positive results should be confirmed by sequencing analysis. Sample analysis may bear the possibility of detecting skin colonisers contaminating blood samples.

Apparatuses and Consumables to be Supplied by the User

The following equipment, consumables and reagents not supplied with this kit are recommended to be used with ***SepsiTest™***.
Do not transfer supplies (e.g., pipettes, microcentrifuges, vortexer, racks) and disposable material as specified by the handlings below from one working place to another.

Sample collection:

- Blood collection tubes, e.g. K-EDTA or citrate S-Monovette®, Sarstedt, Germany

Sample preparation:

- 1x thermomixer (2.0ml tubes), e.g., Eppendorf comfort, Eppendorf, Germany
- 1x cooling rack for 1.5ml tubes (-15 to -25°C)
- 1x vortexer, e.g., VWR, Darmstadt, Germany
- 1x bench top microcentrifuge (≥12,000x*g*), e.g., miniSpin, Eppendorf, Germany
- 1x UV laminar flow hood (Class II)
- Positive sample control (run control), Accurun® 500, Seracare Life Sciences, USA (A500-2503)
- A set of precision pipettes: up to 10µl, up to 20µl, up to100µl, up to 200µl and up to 1000µl, e.g., Eppendorf, Germany
- Sample racks

PCR amplification:

- 1x UV workstation, e.g., Airclean 600, Starlab, Germany
- 1x low speed mini-centrifuge (≤2000x*g*) e.g., MiniFuge, VWR, Darmstadt, Germany
- 1x vortexer, e.g., VWR, Darmstadt, Germany
- 2x cooling racks for 1.5ml tubes (-15 to -25°C)
- 3x cooling racks for 0.2ml PCR tubes (-15 to -25°C)
- PCR cycler, Mastercycler Gradient, Eppendorf, Germany (5331 000.010); other cyclers have to be validated by using positive PCR controls P1 and P2 according to the instructions (pages 24 to 27)
- 2-3x sets of precision pipettes: up to 10µl, up to 20µl, up to100µl, up to 200µl and up to 1000µl, e.g., Eppendorf, Germany

Agarose gel electrophoresis analysis:

- Pre-cast gels (2%) unstained, e.g., Reliant® Gel System, Lonza, USA; alternatively prepare a 2% (w/v) agarose gel (e.g., LE agarose, Biozym, Germany) in 1x TAE buffer
- 1 electrophoresis chamber (15 x 34cm, 1.5l buffer volume capacity)
- Running buffer TAE (50x concentrated), e.g., Biozym, Germany
- An electrophoresis chamber with the following characters: 15 x 34cm, buffer volume capacity: 1.5 litres
- An electrophoresis power supply (300V, 500mA), e.g., Consort E835, Sigma-Aldrich, USA
- A gel documentation system, e.g., E.A.S.Y B-455-F, Herolab, Germany
- A set of precision pipettes: up to 10µl, up to 20µl, up to100µl, up to 200µl and up to 1000µl, e.g., Eppendorf, Germany

Sequencing:

- A DNA sequencing apparatus, e.g., DNA Analyzer ABI 3730XL
- Purification of amplicons, Qiagen, QIAquick® PCR Purification Kit (28104)
- Sequencing, e.g., BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Foster City, CA, USA (optional: use an overnight sequencing service, e.g. GATC Biotech AG, Konstanz, Germany)
- A set of precision pipettes: up to 10µl, up to 20µl, up to100µl, up to 200µl and up to 1000µl, e.g., Eppendorf, Germany

Plastic Consumables and reagents:

- Pipette tips (with aerosol filter), Biosphere®, Sarstedt, Germany
  - 10µl type Eppendorf (70.1115.210)
  - 100µl type Eppendorf (70.760.212)
  - 300µl type Eppendorf (70.765.210)
  - 1000µl type Eppendorf (70.762.211)
- 1.5ml micro tubes, Biosphere®, Sarstedt, Germany (72.706.200)
  - For the preparation of the positive PCR control P2
  - For gel electrophoresis
- PCR tubes, MultiPly®-µStripPro, Sarstedt, Germany (72.990)
- DNA decontamination, e.g., DNA Exitus®, Applichem, Germany (A7089,0100),
- Sterile disposables
  - Gloves, e.g., Kimberly-Clark, Germany
  - Sleeves, e.g., Cardinal Health, Ireland
  - Bouffant Covers, e.g., VWR, Germany
  - Overshoes, e.g., hygi, Germany
- Waste containers for plastics and liquid waste, autoclavable, for each working place.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable sleeve covers, disposable gloves and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDS) which are available on request.

CAUTION: Never add hypochlorite (bleach) or acidic solutions directly to the sample-preparation waste.

Buffers *CM* and *CS* contain guanidinium hydrochloride and guanidinium thiocyanate, respectively, which can form highly reactive compounds and toxic gases when combined with hypochlorite or other acidic solutions. If liquid containing these buffers is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 70% (v/v) ethanol. This kit is to be used only by skilled personnel trained for handling infectious material and molecular biological methods. To avoid false analytic results by DNA contamination of reagents and infection of the user by infectious agents during handling, always wear sterile protective gloves, sterile disposable sleeve covers, a lab coat, sterile protective goggles and disposable overshoes. Work in a laminar flow hood (Class II) irradiated with UV before starting according to the instruction manual of the manufacturer. The UV lamp must be switched off during working. Follow the instructions of the manufacturer for maintenance of the workstation. Dispose potentially infectious material and the waste of the sample preparation (part 1, page 17 to 21) according to the national directive of the health organisation (e.g., Richtlinie über die ordnungsgemäße Entsorgung von Abfällen aus Einrichtungen des Gesundheitsdienstes, 2002).
Separate *Material Safety Data Sheets* are available on request. Molzym reserves the right to alter or modify the product to improve the performance of the kit.

Risk and Safety Phrases

Buffer *CM*
Contains guanidine hydrochloride (>25%): **Harmful, irritant**.
Risk and safety phrases*(page 9): **R22-36/38**, **S26**

Buffer *RL*
Contains sodium azide (<1%): **Harmful**.
Risk and safety phrases*(page 9): **R22-32**, **S28-45-60-61**

Proteinase K
Contains *Proteinase K* (≥1%): **Harmful, irritant**.
Risk and safety phrases*(page 9): **R36/37/38-42, S24-26-36/37**

Buffer *CS*
Contains guanidine thiocyanate (>25%): **Harmful**.
Risk and safety phrases*(page 9): **R20/21/22-32-52/53, S13-61**

Buffer *AB*
Contains isopropanol (<80%): **Highly flammable and irritant**.
Risk and safety phrases*(page 9): **R11-36-67, S7-16-24/25-26**

Buffer *WB*
Contains isopropanol (≤40%): **Flammable and irritant.**
Risk and safety phrases*: **R10-36-67**, **S7-16-24/25-26**

Buffer *WS*
Contains ethanol (<70%): **Flammable**.
Risk and safety phrases*: **R10, S7-16**

***2-mercaptoethanol* (*ß-mercaptoethanol*):**
Poisonous, irritating, environmental hazardous

Directive 67/548/EWG and 1999/45/EG
Risk and safety phrases*: **R23/24/25-38-41-50/53, S26-36/37/39-45-61**

Regulation (EC) No. 1272/2008

Danger

Hazard and precautionary statements**: **H227-H301-H310+H330-H315-H318-H410; P273-P301+P310-P302+P352-P304+P340-P305+P351+P338**

Emergency information (24-hours service)
Emergency medical information in English, French, and German can be obtained 24 hours a day from: Poison Information Centre Mainz, Germany; Tel: +49-6131-19240
Outside of Germany: Please contact the regional company representation in your country.

* **R10**: Flammable; **R11**: Highly flammable; **R20/21/22:** Harmful by inhalation, in contact with skin and if swallowed; **R22:** Harmful if swallowed, **R23/24/25**: Toxic by inhalation, in contact with skin and if swallowed; **R32**: Contact with acids liberates very toxic gas; **R36:** Irritating to eyes; **R36/37/38**: Irritating to eyes, respiratory system and skin; **R36/38:** Irritating to eyes and skin; **R38:** Irritating to skin; **R41:** Risk of serious damage to eyes **R42**: May cause sensitization by inhalation; **R50/53**: Very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment; **R52/53**: Harmful to aquatic organisms, may cause long-term adverse effects in the aquatic environment; **R67:** Vapours may cause drowsiness and dizziness;

S7: Keep container tightly closed; **S13**: Keep away from food, drink and animal feed; **S16:** Keep away from sources of ignition - No smoking; Avoid contact with skin; **S24/25:** Avoid contact with skin and eyes; **S26**: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; **S28**: After contact with skin, wash immediately with plenty of water, **S36/37:** Wear suitable protective clothing and gloves; **S36/37/39**: Wear suitable protective clothing, gloves and eye/face protection; **S45**: In case of accident or if you feel unwell seek medical advice immediately (show the label where possible); **S60:** This material and its container must be disposed of as hazardous waste; **S61**: Avoid release to the environment. Refer to special instructions/safety data sheet.

** **H227**: Combustible liquids; **H301**: Toxic if swallowed; **H310+H330**: Fatal if swallowed or in contact with skin; **H315**: Causes skin irritation; **H318**: Causes serious eye damage; **H410**: Very toxic to aquatic life with long lasting effects;
P273: Avoid release to the environment; **P301+P310:** IF SWALLOWED: Immediately call a POISON CENTER or doctor/physician; **P302+P352:** IF ON SKIN: Wash with plenty of soap and water; **P304+P340:** IF INHALED: Remove to fresh air and keep at rest in a position comfortable for breathing; **P305+P351+P338:** IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

Introduction

System Description

Sepsis is a major cause of death worldwide. Accordingly, there is an urgent need to reduce the uncertainty and time of today's diagnosis in order to reduce mortality of septic patients. Molecular biological techniques, in particular PCR-based methods, are generally accepted as a promising means of early sepsis diagnosis.

SepsiTest™ enables the detection of bacterial and fungal pathogens on a molecular level by the amplification of target sequences of rRNA genes. ***SepsiTest™*** can identify pathogens up to days earlier than culture and in patients who are negative with culture (Orszag et al. 2013).

Molzym's technology of degradation of human DNA and isolation of pathogen target DNA from human samples is combined with universal rDNA PCR assays providing a high quality, straight forward kit for molecular pathogen detection.

By the enzymatic degradation of human DNA, pure pathogen DNA is provided to the assays, minimising false results from unspecific primer binding. Furthermore, the high quality of all reagents of the kit guarantees tolerable reagent-borne false positive signalling (≤3%) under the precautions of the avoidance of DNA contamination.

SepsiTest™ allows the detection of essentially all bacterial and fungal pathogens, including both culturable and non-culturable strains. This is due to the amplification of 16S rDNA (bacteria) and 18S rDNA sequences (fungi) using universal primers. Sequence analysis of amplicons using primers provided with this kit is a confirmation of PCR results and allows the identification of strains detected in samples by BLAST analysis (www.sepsitest-blast.net).

Test Principle

In its concept, ***SepsiTest™*** is a means of molecular analysis of EDTA whole blood for the presence of pathogens. The system combines new solutions for sample preparation and PCR analysis of clinical specimens (Fig. 2, page 12). The procedure includes DNA extraction of samples and PCR or Real-Time PCR analysis using primers targeting conserved regions of the 16S and 18S rRNA genes of bacteria and fungi, respectively. Amplicons are detected by agarose gel electrophoresis. As clinical data indicate, blood samples are advised to be processed and analysed in duplicate for higher sensitivity of detection.

SepsiTest™ is based on two basic steps:

i) Pathogens are enriched from the sample after the degradation of the human DNA and microbial DNA is purified by removal of PCR inhibitors.

ii) The eluate is analysed by universal rDNA PCR for pathogen DNA. Sequence analysis of amplicons together with BLAST search leads to the identification of pathogens.

Part 1: Pre-Analytics

Molzym has developed a technology enabling the enrichment and isolation of pure bacterial and fungal DNA from whole blood samples for PCR analysis. The procedure comprises protocols for the pre-treatment of samples, including the degradation of DNA from lysed human cells, followed by the broad-range lysis of potentially present Gram-negative and Gram-positive bacteria as well as fungi.

The procedure in more detail (Fig. 1). A chaotropic buffer is added to the sample which selectively lyses the human cells, and the nucleic acids released are degraded by added *MolDNase*. After enrichment by centrifugation, pathogens are treated by a reagent, *BugLysis*, which degrades the cell walls of bacteria and fungi. Protein degradation by *Proteinase K* and protein denaturation by a chaotropic buffer finalise the extraction protocol. Finally, pathogen DNA is isolated by a bind-wash-elute procedure using Molzym's CCT technology which enables the recovery of femtogram to picogram amounts of DNA in a 100µl eluate.

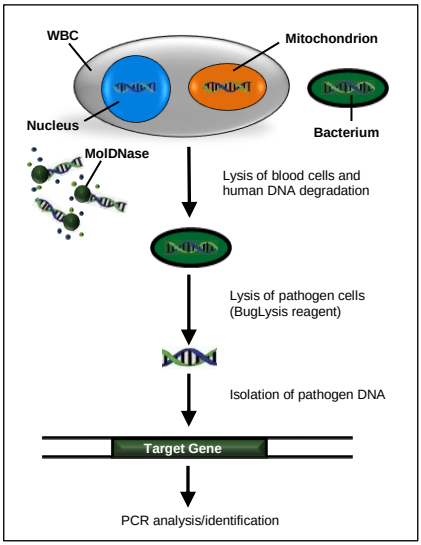


Fig. 1 The principle of testing for bacterial and fungal DNA in blood samples by ***SepsiTest™***.

Part 2(a): PCR Analytics

DNA eluates (part 1, page 10) are used for broad-range 16S and 18S rDNA PCR analysis. Two assays are supplied, assay *MA Bac* (bacteria) and assay *MA Yeasts* (fungi) enabling the sensitive detection of pathogens. A protocol for the detection of amplicons by agarose gel electrophoresis is supplied. Real-Time PCR is another option. To test for the performance of the assay regarding PCR inhibition by DNA extracts, an internal control assay (*MA IC*) is included in the kit.

Part 2(b): Sequencing

A protocol for sequence analysis of amplicons is supplied in order to identify detected pathogens. The procedure includes a short protocol for amplicon purification and another for sequencing of amplicons. Primers for sequencing are supplied with this kit.

This kit is marked according to European directive 98/79/EC **for *in-vitro* diagnostic use**. Many routine applications demand high sensitivity and specificity analysis of bacterial and fungal pathogens in clinical specimens. The analysis of specimens using ***SepsiTest™*** is a two step generation of data (Fig. 2). After sample extraction, at the first step negative or positive results are obtained by PCR or Real-Time PCR analysis, indicating the absence or presence of bacterial and fungal target sequences in the sample. If positive, the second step of the analysis encompasses sequence analysis of amplicons. Sequencing is a way of confirming a positive PCR result and of gaining information on the identity of an organism detected. Therefore, sequence analysis should always follow PCR detection of amplicons. Sequencing of amplicons from assay *MA Bac* (bacteria) employs primers, *SeqGN16* and *SeqGP16*. Although not strictly discriminative, *SeqGN16* targets mainly Gram-negative and *SeqGP16* mainly Gram-positive bacteria. By using both sequencing primers in separate reactions, mixed strains of the two groups can be resolved. Sequencing of amplicons from the fungal assay, *MA Yeasts*, is performed using *SeqYeast18*. Bacterial taxa are identified with the help of the free online tool, SepsiTest™-BLAST (www.sepsitest-blast.net). There are three potential outcomes of the sequencing analysis:

i) In case of readable sequences (Fig. 2, A, readable; below), the BLAST online search results in the identification of strains at the species (99 to 100% sequence identity) or genus level (>97% sequence identity), depending on the quality of the read.

ii) If SepsiTest™-BLAST analysis indicates poor quality, overlapping sequences may be the reason as a result of more than one target sequence present in the reaction (Fig. 2, B, not readable); in such cases a service is available for identification of bacteria in mixed infections (www.ripseq.com).

iii) If the amount of amplicon is too low for a sequencing reaction (Fig. 2, C, no read), the result is considered negative (below the limit of sequencing detection). In this case, another analysis of a new sample should be performed, if possible.

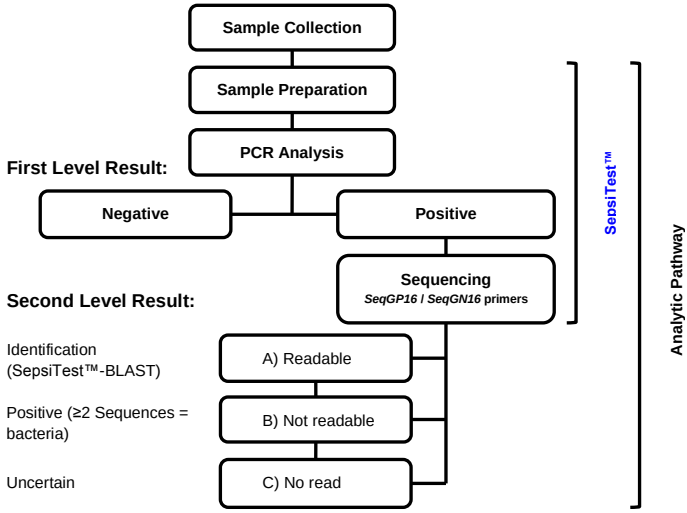


Fig. 2: Detection of microorganisms in samples using ***SepsiTest™*** and sequencing analysis. The analytic pathway includes the detection and identification of bacteria and fungi (only bacteria shown).

Controls and Validation

Controls

A series of controls should be routinely performed to test the performance of the kit. Below a list of controls is given and commented. More information on the exact procedures for running controls are given in the respective sections.

Sample Controls

Positive Sample Control (Run Control)

This control reflects the performance of the extraction procedure and should be performed at least once per setup. There are two ways suggested to perform a run control:
i) Negative samples (e.g., blood or sterile 0.9% sodium chloride) are spiked with 100 to 1000cfu/ml of a cultured Gram-negative (e.g., *E. coli*), Gram-positive (e.g., *S. aureus*) and fungal pathogen (e.g., *C. albicans*), respectively, and run through the extraction protocol followed by analysis as described in this kit.
ii) The extraction is performed using a commercial standard. Molzym has evaluated Accurun® 500 (SeraCare Life Sciences, USA).

Negative Sample Control

This test should be run together with the positive sample control to test for potential cross-contamination during sample extraction. For this, a negative blood sample is used and run through the extraction and detection protocols of this kit.

PCR Controls

Positive PCR Control

This test includes a definite number of target sequence copies to make sure that the assay is performing as specified. The set of controls comprises of a high (P1) and low (P2) standard DNA for Mastermix Assay Bacteria (*MA Bac*) and Mastermix Assay Yeasts (*MA Yeasts*). The high concentrated DNA standard (P1) is supplied with this kit and has a concentration of 0.1 to 1.0ng target DNA/µl. Using this standard DNA indicates the functioning of the assays. The low concentrated DNA standard (P2) is diluted from P1 to 0.2 to 2.0pg/µl and constitutes a multiple of the lower limit of detection being a test for the sensitivity of the assays. Positive PCR controls P1 and P2 have to be performed with each set of analyses, i.e. with *MA Bac* and *MA Yeasts*.

Prepare the positive PCR control at a place where DNA is handled. Thaw DNA Standard P1 and DNA dilution buffer (Kit 4B). Vortex the P1 vial and pulse centrifuge. Pipette 998µl of DNA dilution buffer in a 1.5ml sterile polypropylene tube, add 2µl DNA Standard P1 and vortex to mix. Store chilled in a cooling rack until use. Always prepare P2 freshly for each series of PCRs. Do not re-use, because dilute DNA solutions tend to be unstable.

Negative PCR Control (Reagent Control)

This setup contains all reagents except that supplied DNA-free water is added instead of eluate (target DNA). The control is meant to detect any exogenous DNA coming in as carry-over or handling contamination during running parallel tests and pipetting of reagents.

Internal PCR Control

Potential inhibition of the PCR reaction by components of the specimens co-purified with DNA is measured by this control. The kit supplies an assay (*MA IC*) including a target DNA sequence to which an aliquot of the sample extract is added. Generation of an amplicon indicates the absence of inhibitory substances.

Validation

Broad-range of Primers

The broad-range binding of the primers to universal sites of the 16S and 18S rRNA genes was analysed with a sequence alignment algorithm, allowing 1 mismatch (excluding terminal sites). As a result, more than 345 species are detectable, among which more than 200 species have been sequence-identified in clinical evaluations so far (Tab. 1, page 15).

List of Strains Tested for Extraction

SepsiTest™ contains a reagent, *BugLysis*, for the degradation of cell walls of Gram-positive and Gram-negative bacteria, and fungi. The reagent has been evaluated with the following clinical strains, using Real-Time PCR for analysis (T_m analysis):

Gram-positive bacteria: *Bacillus cereus, B. subtilis, Corynebacterium diphteriae, Enterococcus faecalis, E. faecium, Lactobacillus sp., Micrococcus luteus, Mycobacterium phlei, Staphylococcus aureus, S. carnosus, S. epidermidis, Streptococcus agalactiae* (Sero-Group B), *S. mutans*, *S. oralis, S. pneumoniae, S. pyogenes* (Sero-Group A), streptococci (Sero-Group G).

Gram-negative bacteria: *Acinetobacter baumannii*, *Escherichia coli, Enterobacter aerogenes, Serratia marcescens, Klebsiella pneumoniae, Neisseria meningitis, N. subflava, Porphyromonas gingivalis, Proteus mirabilis, P. vulgaris, Pseudomonas aeruginosa, P. stutzeri, Stenotrophomonas maltophilia.*

Fungi: *Aspergillus fumigatus, Candida albicans, C. glabrata, C. krusei, C. parapsilosis, C. tropicalis, Cryptococcus neoformans*.

Analytical Specificity

SepsiTest™ includes two assays, one for the general detection of bacteria (*MA Bac*) and another for the detection of fungi (*MA Yeasts*). Experiments including yeast DNA in assay *MA Bac* and bacterial DNA in assay *MA Yeasts* gave no indication of cross reactivity of the primer pairs with the unspecific DNA. Cross reactivity was shown for bacterial primers used in assay *MA Bac* with a large excess of human DNA (see also Mühl et al. 2010). This problem is addressed by the pre-analytical treatment of samples to remove human DNA (‘Test Principle’, pages 10 to 12).

Analytical Sensitivity

Molzym’s sample pre-treatment and DNA isolation constitutes the optimal solution for high sensitivity PCR and Real-Time PCR analysis of DNA from pathogenic bacteria and fungi. By this combination, for instance, *S. aureus* can be detected at 20cfu/ml blood by the universal PCR assay. For other strains, see Tab. 2 (page 15) and Mühl et al. (2010). Spiking experiments using negative samples and serial dilutions of cultured strains of clinical isolates showed the detection limits indicated in Tab. 2, page 15.

Clinical Evaluation

A broad spectrum of Gram-negative, Gram-positive and fungal organisms were identified in clinical samples using ***SepsiTest™*** (Tab. 1, page 15).

Tab. 1: Microorganisms identified in clinical evaluations.

Gram-negative bacteria		
Acinetobacter spp.	*Pseudoxanthomonas spadix*	*Listeria monocytogenes*
Actinomyces sp.	*Ralstonia pickettii*	*Microbacterium aurum*
Aeromonas veronii	*Raoultella planticola*	*Micrococcus spp.*
Bacteroides fragilis	*Schlegelella aquatica*	*Mycetocola sp.*
Bartonella quintana	*Serratia spp.*	*Mycobacterium spp.*
Bordetella petri	*Sphingomonas sp.*	*Mycoplasma sp.*
Borrelia garinii	*Spirosoma rigui*	*Nocardia sp.*
Bradyrhizobium sp.	*Shigella flexneri*	*Paenibacillus sp.*
Burkholderia fungorum	*Stenotrophomonas maltophilia*	*Parvimonas micra*
Campylobacter coli	*Tepidimonas thermarum*	*Peptoniphilus harei*
Candidatus Neoehrlichia	*Variovorax sp.*	*Peptostreptococcus stomatis*
Citrobacter freundii	*Veillonella sp.*	*Planomicrobium okeanokoites*
Cloacibacterium normanense	*Weeksella sp.*	*Propionibacterium acnes*
Comamonas testosteroni	*Zoogloea sp.*	*Rothia spp.*
Coxiella burnetii		*Ruminococcus productus*
Dialister invisus	**Gram-positive bacteria**	*Staphylococcus spp.*
Edwardsiella tarda	*Actinomyces sp.*	*Streptococcus spp.*
Enhydrobacter aerosaccus	*Aerococcus urinaeequi*	*Tropheryma whippleii*
Enterobacter spp.	*Anaerococcus spp.*	*Vagococcus carniphilus*
Escherichia spp.	*Bacillus spp.*	
Fusobacterium nucleatum	*Bifidobacterium spp.*	**Fungi**
Haemophilus spp.	*Brevibacterium spp.*	*Aspergillus spp.*
Helicobacter pylori	*Carnobacterium viridans*	*Candida spp.*
Hyphomicrobium facile	*Clostridium spp.*	*Cladosporium cladosporioides*
Janthinobacterium lividum	*Corynebacterium spp.*	*Cryptococcus spp.*
Klebsiella spp.	*Dolosigranulum pigrum*	*Didymella exitialis*
Lautropia mirabilis	*Enterococcus spp.*	*Davidiella tassiana*
Leptotrichia sp.	*Eremococcus coleocola*	*Malassezia spp.*
Methylobacterium sp.	*Exiguobacterium sp.*	*Peniophora nuda*
Moraxella spp.	*Facklamia spp.*	*Saccharomyces cerevisiae*
Morganella morganii	*Finegoldia magna*	*Schizophyllum radiatum*
Neisseria spp.	*Gemella spp.*	*Sistotrema brinkmannii*
Parabacteroides distasonis	*Granulicatella adiacens*	*Sporobolomyces sp.*
Paracoccus aminovorans	*Janibacter sp.*	*Udeniomyces pannonicus*
Petrobacter sp.	*Jeotgalicoccus pinnipedialis*	
Proteus spp.	*Kocuria spp.*	**Protist**
Providencia stuartii	*Lactobacillus spp.*	*Plasmodium falciparum*
Pseudomonas spp.	*Lactococcus lactis*	
	Leifsonia sp.	

Tab. 2: Analytical sensitivity of ***SepsiTest™***.
Minimum titre resulting in positive results from 3 repeated extractions in whole blood samples spiked with strains. Analysis: Real-Time PCR (5µl eluate/assay; Assays: *MA Bac* and *MA Yeasts*) with melting curve analysis.

Strain	cfu/ml detected
Gram-positive bacteria	
Enterococcus faecalis	20
Staphylococcus aureus	20
Staphylococcus epidermidis	20
Streptococcus pneumoniae	75
Gram-negative bacteria	
Escherichia coli	40
Klebsiella pneumoniae	50
Moraxella catarrhalis	50
Pseudomonas aeruginosa	80
Fungi	
Candida albicans	10
Candida glabrata	10

Avoidance of DNA Contamination

Care for the avoidance of DNA contamination from exogenous sources should be taken during the complete pathway from sample collection to analysis. Also, it is important to minimise cross-contamination from sample to sample. For guidance see Roth et al. (2001) and Espy et al. (2006) and DIN 58967-60 (Oktober 2001). A short summary of precautions is given below:

- The guidelines of the national health organisations, e.g., Robert-Koch-Institute (Germany), for sample collection, including sterilisation of the skin should be followed.
- Generally, for pre-analytical and analytical processing, use places decontaminated from DNA. We recommend to perform handling steps under UV-irradiated workstations. UV irradiation must be done before working according to the recommendations of the manufacturer. Routinely treat the surfaces of the working places with a commercial DNA decontamination reagent which is compatible with sterile protective gloves. Make sure that the material to be decontaminated is resistant to such treatment. Do not transfer supplies (e.g., pipettes, microcentrifuges, vortexer) and disposable material as specified by the handlings below from one working place to another. Each working place should be equipped with refrigerators (+4 to +12°C) and freezers (-15 to -25°C) for storage of the reagents of the kit. For transport of vials with mastermixes from one place to another, cooling rack should be used and a separate decontaminated with a decontamination reagent, e.g., DNA Exitus® after each transport.
- Handle potentially infectious material with great care under a laminar flow hood (Class II) in order to protect yourself from infection, and avoid cross-contamination of samples and carry-over contamination of extraction buffers and reagents.
- Wear sterile protective gloves and sterile disposable sleeve covers at any handling step, including handling of infectious material, sample pre-treatment and PCR analysis. Frequently change protective gloves during handling. Use protective goggles, and disposable lab coats and overshoes and change when moving from one lab to another (below).
- Take care to maintain a DNA-free environment during opening the vials and bottles and handling the mastermixes. Close vials and bottles immediately after the removal of liquid.
- All DNA extraction buffers and reagents (DNA Isolation unit, kits 1 and 2) are assembled as 4 sample extraction units to minimise the risk of contamination of buffers and reagents during working with samples. Opened vials can be used within 4 days when stored under the conditions specified.
- Use only DNA-free pipette tips, vials and consumables recommended (page 7).
- Generally, run PCR negative and positive controls as well as internal controls with each series of assays to check for DNA contamination by handling during the preparation of mastermixes and the correct performance of the assays, respectively.

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Part 1: Pre-Analytics

DNA Isolation

Use the following kits:

- ***Kit 1*** *(store at +18 to +25°C)*
  - *Buffers (bag A)*
  - *ST - Sample tubes (bag B)*
  - *SC - Spin columns (bag C)*
  - *CT - Collection tubes (bag C)*
  - *ET - Elution tubes (bag C)*
- ***Kit 2*** *(store at -15 to -25°C)*
  - *Enzymes and Reagents*

Part 1 - Pre-Analytics - DNA Isolation

Sample Collection

Because of the universal nature and the extreme sensitivity of detection of the assay, special care has to be taken for sample collection to avoid contamination by skin and environmental microorganisms. Transfer the samples to the laboratory for immediate processing (pages 17 to 37). Alternatively, store the samples in a refrigerator (+4 to +12°C). The stored sample must be analysed within 2 days after sample collection. For longer storage conditions, see the appropriate section (page 20, step 5) of the DNA isolation protocol.

Isolation of Pathogen DNA

Work in a place, which is ideally in a lab separated from places where mastermixes are handled and PCR reactions are performed. Calibrate the procedure by spiking negative samples (e.g., blood or sterile 0.9% sodium chloride) with dilutions of full-grown cultures of pathogens or by using Accurun® 500 standard ('positive sample control', page 13). Please call Molzym for more information about positive sample controlling.

! For equipment, consumables and reagents to be supplied by the user see pages 6 to 7.

! As clinical results show, patient samples, if possible, should always be processed in duplicate to reach full sensitivity of pathogen detection using this kit.

! To minimise cross-contamination, this unit is assembled in vials containing buffers or reagents for 2 blood sample extractions in duplicate. Once removed for usage and opened, store buffers in a DNA-free environment at room temperature (+18 to +25°C) in the dark for up to 4 days. To avoid carry-over contamination, close caps of vials immediately after removal of solution.

! Take care that *MolDNase B, BugLysis, ß-mercaptoethanol* and *Proteinase K* solutions (Kit 2) are placed in a cooling rack adjusted to -15 to -25°C. After use, close caps of vials and replace vials to the freezer (-15 to -25°C).

Caution: ß-mercaptoethanol is toxic. Take care not to inhale and otherwise come into contact with.

! Adjust the thermomixer to 37°C (needed for step 7, page 20).
Place vials of buffer *ES* (100µl for each sample) into the thermomixer (needed for step 15, page 21).

! Use only fresh EDTA or citrate-stabilised blood. After collection, the blood container should be transported to the laboratory and processed immediately. If this is not possible, the blood container should be placed in a refrigerator (+4 to +12°C), where it can be stored for 2 days at maximum. ***Do not freeze samples*** to avoid potential loss of pathogen DNA due to cell disruption as a result of freezing and thawing. If freezing of samples is desired, use Molzym's ***UMD-Tubes*** (order no. Z-801-020) which stabilise blood samples. Thaw samples to room temperature (+18 to +25°C) for extraction. You can also interrupt the following procedure of pathogen DNA isolation at a certain step (step 5, page 20).

! Per duplicate of a sample, mark a *Spin column* (*SC*), two *Collection tubes* (*CT*) and one *Elution tube* (*ET*) of Kit 1 with a permanent marker for identification of the sample.

! Leave items used for the workflow (e.g., pipettes, racks, pipette boxes) in the workstation and have them exposed to the UV irradiation for decontamination before starting. In case of contamination of pipettes and other items or spilling the surface of the workstation by sample material, decontaminate as advised (Safety Information, page 8). Arrange all items according to your personal customs.

Protocol

Caution: Wear sterile protective gloves, sterile disposable sleeve covers, a disposable lab coat and protective goggles when handling infectious material. Work in a laminar flow hood (Class II) irradiated with UV before starting according to the instructions of the manufacturer. Follow the instructions of the manufacturer for maintenance of the workstation. Use fresh pipette tips with each pipetting step.

1A) How to Start

- **Kit 1** contains buffers and consumables (single bags A to C) for the extraction and isolation of DNA from blood samples. Open the bags and vials only in the laminar flow hood (Class II). Shortly centrifuge the vials and arrange in a separate rack according to the sequence of steps as below:

CM – DB1 – RS – RL – RP – CS – AB – WB – WS – ES →

Excess vials/consumables of the opened bags can be stored at room temperature (+18 to +25°C) in a dark, DNA-free place for 4 days.

- **Kit 2** contains the enzymes and reagents. Take care that *MolDNase B*, *BugLysis*, *β-mercaptoethanol* and *Proteinase K* solutions are kept at -15 to -25°C throughout. Replace enzymes and the reagent to the freezer (-15 to -25°C) immediately after handling.

1B) Arrangements and Pre-Treatment of Samples

Whole blood (EDTA or citrate-stabilised)

Per sample, arrange and mark a *Sample tube* (*ST* tubes, Kit 1, bag B) in another rack and pipette 1ml fresh whole EDTA- or citrate-stabilised blood into the *ST* tube. Continue with section 1C step 1, page 20.

Because of the low concentration of pathogens in the blood (Wellinghausen et al., 2009), samples should be extracted and analysed in duplicates of 1ml for high sensitivity of detection

1C) DNA Isolation Protocol

1. **Pipette 250µl buffer *CM* to each *ST tube* containing 1.0ml blood sample (section 1B, page 19). Vortex at full speed for 15s to mix. Let stand on the bench at room temperature (+18 to +25°C) for 5min.**

   Buffer *CM* is a chaotropic buffer that lyses the human cells.

   Caution: Buffer *CM* is an irritant. Avoid contact with skin and eyes.

2. **Briefly centrifuge to clear the lid. Pipette 250µl buffer *DB1* to the *ST tube*. Thereafter pipette 10µl *MolDNase B* to the lysate in the *ST tube*. Immediately vortex for 15s and let stand for 15min at room temperature (+18 to +25°C). Replace the vial with *MolDNase B* to -15 to -25°C for further storage.**

   During this step the nucleic acids released from human cells are degraded.

3. **Centrifuge the *ST tube* in a bench top microcentrifuge at ≥12,000x*g* for 10min. Thereafter, carefully remove the supernatant by pipetting and discard.**

   Human cell debris and potentially present pathogen cells are sedimented.

4. **Pipette 1ml buffer *RS* to the sediment and resuspend by vigorous vortexing.** Depending on the sample, the pellet may be rigid and resuspension may take some time. Optionally stir the sediment with the pipette tip and pipette in and out until resuspended.

5. **Centrifuge the *ST tube* in a bench top microcentrifuge (≥12,000x*g*) for 5min. Carefully remove the supernatant by pipetting and discard.**

   This washing removes residual *MolDNase B* activity, chaotrope and part of the PCR inhibitors.

At this point the procedure can be interrupted by freezing the sample (-15 to -25°C). For further processing, thaw the sample at room temperature (+18 to +25°C) and proceed with step 6.

6. **Pipette 80µl buffer *RL* to the *ST tube*. Resuspend the sediment by vigorous vortexing. Briefly centrifuge to clear the lid.**

7. **Pipette 20µl *BugLysis* directly into the extract in the *ST tube* and then add 1.4µl *ß-mercaptoethanol*. Vortex the tube for 15s and incubate in a thermomixer at 37°C and 1,000rpm for 30min. Store non used *BugLysis in the* vial at -15 to -25°C for up to 4 days.**

   The cell walls of potentially present bacteria and fungi are degraded.

   Caution: ß-mercaptoethanol is toxic. Take care not to inhale and otherwise come into contact with.

   After the incubation, adjust the thermomixer to 56°C.

8. **Briefly centrifuge the *ST tube*. Add 150µl buffer *RP* to the *ST tube*. Then pipette 20µl *Proteinase K* directly to the extract in the *ST tube*. Vortex the *ST tube* for 15s and incubate at 56°C and 1,000rpm for 10min. Store non used *Proteinase K* in the vial at -15 to -25°C for up to 4 days.**

   After the incubation, adjust the thermomixer to 70°C (make sure that the vials of buffer *ES* are placed in the mixer, needed at step 15).

9. **Briefly centrifuge the *ST tube* and pipette 250µl buffer *CS* into it. Vortex tube at full speed for 15s.**

   Cells are lysed and protein is denatured.

10. **Briefly centrifuge the *ST tube*. Pipette 250µl binding buffer *AB* to the *ST tube*. Vortex the tube at full speed for 15s.**

11. **Briefly centrifuge the *ST tube* and transfer the lysate to a *Spin column* (*SC*; Kit 1, bag C) pre-assembled in a 2.0ml *Collection tube* (*CT*) by pipetting. Close the lid of the *Spin column* and centrifuge at ≥12,000x*g* for 30s (or minimum time of the centrifuge, e.g. 60s).**

    At this point nucleic acids bind to the matrix.

12. **Remove the closed *Spin column* from the centrifuge. Open the lid, remove the *Spin column*, discard the *Collection tube* with flow-through and replace the column into a new 2.0ml *Collection tube* (*CT*; Kit 1, bag C). Pipette 400µl buffer *WB* to the *Spin column*. Close the lid and centrifuge at ≥12,000x*g* for 30s (or minimum time of the centrifuge, e.g. 60s).**

13. **Remove the closed *Spin column* from the centrifuge. Open the lid, remove the *Spin column*, discard the *Collection tube* with flow-through and replace the column into a new 2.0ml *Collection tube*. Pipette 400µl of buffer *WS* to the *Spin column*. Close the lid of the *Spin column* and centrifuge at ≥12,000x*g* for 3min.**

    This step removes salts and dries the column matrix.

14. **Carefully remove the closed *Spin column* from the centrifuge. Avoid splashing of the flow-through to the *Spin column*. Remove the *Spin column* from the *Collection tube* and place into a 1.5ml *Elution tube* (*ET*; Kit 1, bag C). Discard the *Collection tube* containing the flow-through.**

15. **Place 100µl buffer ES (vials in the thermomixer is already preheated to 70°C), in the center of the column, close lid and incubate for 1min at room temperature (+18 to +25°C). Thereafter, centrifuge at ≥12,000xg for 1min to elute the DNA. Finally, remove the *Spin column* from the *Elution tube* and close the lid. Discard the *Spin column*.**

16. **Store the *Elution tube* containing the eluate at +4 to +12°C if analysed at the same day or freeze at -15 to -25°C until further use (part 2, pages 22 to 31).**

    Avoid frequent freeze-thaw cycles, because this may result in a decay of the eluted DNA (in particular at low DNA concentrations).

Part 2: Analytics

PCR Detection and Sequence Identification

Protocols for PCR, Gel Electrophoretic Analysis & Sequencing

Addendum:
Real-Time PCR Protocols

Use the following kits of the PCR Detection and Identification Unit:

Kit 3
PCR Reagents (store at -15 to -25°C)

Kit 4
PCR Controls and Detection Reagents (store at -15 to -25°C)

Consumables PCR Detection and Sequencing *(store at +18 to +25°C)**:***
– MT - Mastermix tubes, 1.5ml for Kit 3 (in Kit 1)
– MT - Mastermix tubes, 1.5ml for Kit 4A (in Kit 1)

Part 2 - Analytics

Description of the Assays

With this unit, PCR assays are supplied for the testing of the presence of bacterial and fungal DNA in extracts of blood samples. The 'Mastermix Assay Bacteria' (*MA Bac*, kit 3) and 'Mastermix Assay Yeasts' (*MA Yeasts*, fungi, kit 3) are based on primers that bind to conserved regions of the 16S and 18S rRNA genes of bacteria and fungi, respectively. The tests comprise a two-step procedure including the use of **i)** the mastermixes *MA Bac* and *MA Yeasts* (Kit 3) for the PCR amplification of sequences using extracted DNA (pages 18 to 21) and **ii)** agarose gel electrophoresis for the detection of amplified DNA, using components supplied with Kit 4. Alternatively, protocols for the Real-Time PCR detection are supplied with the Addendum (page 32 to 37).
MA Bac and *MA Yeasts* are 2.5x concentrated solutions, the final volume of the reaction mixture being 25µl. This PCR Detection unit contains all components necessary for PCR runs.

The 'Mastermix Assay Internal Control' (*MA IC*, kit 4) is a control for the performance of the PCR assay and includes an amplifiable target DNA. Potential inhibition of the PCR reaction by components of the specimens co-eluted with DNA is measured by *MA IC*.

Protocols for amplification are supplied for the following instruments:

- Thermal Cycler (protocol, page 27):
  - Mastercycler Eppendorf
- Real-Time PCR instruments (addendum pages 33 to 37):
  - LightCycler® 1.5, 2.0, 96, 480 and Nano, Roche
  - DNA Engine Opticon®, BioRad
  - Mx3000P®, Stratagene
  - ABI 7500 Fast®, Life Technologies
  - Rotor-Gene®, Qiagen

If using other instruments, make sure that the Assays *MA Bac* and *MA Yeasts* perform correctly with the cycler. For this purpose, perform PCR reactions using PCR DNA Standard P1 and P2 which both should result in an amplification product. See PCR Detection for the procedure (pages 25 to 30)

Packaging, Storage and Handling

The purification and packaging of the mastermixes supplied in this PCR detection unit are performed under standard precautions for the avoidance of air-borne and handling-based DNA contaminations. The mastermixes are supplied as a 2.5x concentrated solution in DNA-free screw cap vials. Store all vials in this unit (Kits 3 and 4) at -15 to -25°C upon receipt. For usage, the mastermixes are thawed at room temperature (+18 to +25°C) and thereafter placed in cooling racks adjusted to -15 to -25°C. After use, the mastermixes of Kits 3 and 4 can be stored in the refrigerator (+4 to +12°C) for further use at the same day, but should be replaced to -15 to -25°C for longer storage.
MolTaq 16S has to be kept at -15 to -25°C throughout handling (cooling rack). Replace *MolTaq 16S* to the freezer (-15 to -25°C) after handling.
It is important to note that the DNA staining solution (*DS*, Kits 3 and 4A) is sensitive to light and should be stored in the dark during handling and storage. ***Once thawed, do not freeze again*** and store at +4 to +12°C for further use.
Store *DNA dilution buffer* and *DNA Standard P1* at +4 to +12°C after thawing.
Take care to maintain a DNA-free environment during opening the vials and handling the mastermixes by working under a UV-decontaminated workstation. Use only certified bacterial DNA-free pipette tips and PCR consumables recommended for running the assays (page 7).

Quality Control and Specifications

Negative PCR controls using supplied DNA-free water instead of eluate are used for routine analysis of contamination by microbial DNA in the purified final mastermixes (*MA Bac* and *MA Yeasts*). Guarantee is given for the absence of signals in negative controls at a rate of ≤3%, provided the avoidance of contamination by handling errors.

Positive PCR controls should always be run and contain a high (P1) and low (P2) concentrated target DNA amount per assay. Standard DNA P1 is supplied with Kit 4B and serves as a run control of the PCR reaction. P2 is diluted from P1 and indicates the sensitivity of the assays, *MA Bac* and *MA Yeasts*. The Standard DNA is a mixture of known amounts of genomic DNA from *Bacillus subtilis* and *Saccharomyces cerevisiae*.

Caution: Wear sterile protective gloves, sterile disposable sleeve covers, a disposable lab coat, protective goggles, a bouffant cover and disposable overshoes during the setup of mastermixes. Work in a PCR workstation irradiated with UV before starting according to the instruction manual of the manufacturer. Follow the instructions of the manufacturer for maintenance of the PCR workstation. Do not work under UV irradiation.

PCR Detection and Identification of Pathogens by Sequencing

PCR Detection

Assays *MA Bac* and *MA Yeasts*:
With each mastermix an extra volume is supplied to run PCR controls. Negative PCR controls should always be performed, at least with 10% of the sample runs, to test for potential cross-contamination or other carryovers resulting from handling or air-borne errors. Also, with each set of sample runs positive PCR controls (page 13) must be included to control the performance of the assay. Follow the instructions for the performance of positive PCR controls (pages 24 to 27).
Assay *MA IC*:
The Assay *MA IC* (internal control; Kit 4A) is a test to check for potential PCR inhibition that should also flank each series of sample testing (pages 24 to 27).
Validity of results:
Only if the negative PCR controls (*MA Bac* and *MA Yeasts*) lack a PCR signal, the positive PCR controls (P1 and P2) and the Assay *MA IC* result in a band of specific size in the gel electrophoresis analysis, the results of the sample test can be considered true results, particularly in the case of the absence of a signal indicating a negative result and thus absence of pathogens in the sample (pathogen load below the limit of detection).

2A) How to Start

! For equipment, consumables and reagents to be supplied by the user see page 7.

Avoidance of DNA Contamination

! To avoid contamination it is important that the setup of *MA Bac* and *MA Yeasts* is performed in a lab separated from the setup of *MA IC* (as well as from DNA extraction and PCR amplifications).

! For each pipetting use fresh tips.

! Take care that all handling is performed in a DNA-free environment.

! To avoid contamination, close caps immediately after removal of solution.

Storage of the PCR Reagents after Handling

! After use, keep the 2.5x mastermixes and H_2O in a refrigerator (+4 to +12°C) if reused at the same day or store at -15 to -25°C for longer periods.

! Replace *MolTaq 16S* in a cooling rack (-15 to -25°C). Always keep and store *MolTaq 16S* at -15 to -25°C. ***Do not interrupt*** the cooling of *MolTaq 16S*.

! After first use, store DNA staining solution (*DS*) in the dark at +4 to +12°C. **Do not re-freeze**.

! After the first use, store *DNA dilution buffer* and *DNA Standard P1* at +4 to +12°C.

! Dilute DNA solutions (P2) tend to be unstable. Always prepare P2 for each series of PCRs (step 14, page 27).

Places where Handlings are performed

Symbols and explanation of the PCR working places:

DNA-free

Work under a PCR UV workstation. Use components of **Kit 3.**
For the preparation of mastermixes *MA Bac* and *MA Yeasts*.

DNA handling

Work under a UV laminar flow hood (Class II), where samples are prepared. Use components of **Kit 4.** For the preparation of:

- Sample loading into the assays
- Assay *MA IC*
- Positive PCR controls P1 and P2

PCR Assaying

Per assay (*MA Bac, MA Yeasts* and *MA IC*), the following PCR reactions have to be run:

DNA-free

MA Bac

- 1 reaction per sample
- 2 reactions for the positive controls (P1, P2)
- 1 reaction for negative control (NC)

MA Yeasts

- 1 reaction per sample
- 2 reactions for the positive controls (P1, P2)
- 1 reaction for negative control (NC)

DNA

MA IC

- 1 reaction per sample (IC)
- 1 reaction for reference (IC_W, template H_2O)

Thaw the following vials at room temperature (+18 to +25°C):

DNA-free

Kit 3:

- H_2O
- 2.5x *MA Bac*
- 2.5x *MA Yeasts*
- *DS;* keep dark

DNA handling

Kit 4A:

- H_2O
- 2.5x *MA IC*
- *DS,* keep dark

Kit 4B:

- *DNA Standard P1*
- *DNA dilution buffer* (for P1)

2B) Setup of the Assays

Keep all PCR tubes filled with PCR-ready mastermix chilled in the cooling racks, until placing in the PCR cycler. Cooling of the PCR tubes is important to avoid the generation of primer dimers.

DNA-free

Preparation of PCR-ready mastermixes *MA Bac* and *MA Yeasts*

1. **Arrange the PCR tubes for *MA Bac* and *MA Yeasts* in a PCR cooling rack (-15 to -25°C) and mark (PCR Assaying, page 25).**
2. **Place *MolTaq 16S* (Kit 3) in a cooling rack (-15 to -25°C).**
3. **Use a *MT* (*Mastermix tube* 1.5ml for Kit 3; in Kit 1) for *MA Bac* and another *MT* tube for *MA Yeasts*. Place the *MT* tubes in a cooling rack. Pipette the supplied components of Kit 3 (Tab. 3, page 27) into each *MT* tube. Vortex the tubes to mix and briefly centrifuge.**
4. **Pipette 20µl of the PCR-ready mastermix *MA Bac* into each PCR tube labelled samples, P1, P2 and NC, respectively. Repeat the procedure with mastermix *MA Yeasts*.**
5. **Add 5µl *H_2O* (DNA-free water; Kit 3) into the NC PCR tube. Close all PCR tubes with the caps.**

DNA handling

6. **Place the PCR tubes in another cooling rack designated for transport to the UV laminar flow hood (Class II).**
7. **Arrange the PCR tubes for *MA IC* in a PCR cooling rack (-15 to -25°C) and mark (PCR Assaying, page 25).**

Sample loading for assays *MA Bac, MA Yeasts* and *MA IC*

8. **For preparation of *MA IC*, pipette 5µl of each sample eluate into an empty PCR tube (IC). The PCR tube for the reference (IC_W) stays empty.**
9. **Pipette 5µl of each sample eluate into the PCR tubes containing mastermix *MA Bac* and *MA Yeasts*, respectively. Close the PCR tubes.**

Preparation of PCR-ready mastermix *MA IC*

10. **Place *MolTaq 16S* (Kit 4A) in a cooling rack (-15 to -25°C).**
11. **Use a *MT* (*Mastermix tube* 1.5ml for Kit 4A; in Kit 1) and place in a cooling rack. Pipette the supplied components of Kit 4A into the tube (Tab. 3, page 27). Vortex the tube to mix and briefly centrifuge.**
12. **Pipette 20µl of mastermix *MA IC* into each PCR tube marked IC (containing eluate) and IC_W (empty).**
13. **Pipette 5µl *H_2O* (DNA-free water; Kit 4A) into the reference tube IC_W.**

    Close the PCR tubes.

Positive PCR controls (*P1* and *P2*)

14. **Vortex the P1 vial and pulse centrifuge. Pipette 998µl of *DNA dilution buffer* into a 1.5ml sterile polypropylene tube (not supplied). Add 2µl *DNA Standard P1* and vortex to mix. Briefly centrifuge.**
15. **Pipette 5µl of positive PCR control P2 into a PCR tube containing *MA Bac* and *MA Yeasts*, respectively. Repeat with P1.**
16. **Continue with section 2C PCR Thermocycling.**

Tab. 3: Preparation of PCR-ready mastermixes (Kits 3 and 4A). Volumes in µl.

reactions	*MA Bac*, *MA Yeasts* or *MA IC*	*H_2O*	*DS*	*MolTaq 16S*
1	10.0	7.5	2.5	0.8
2	20.0	15.0	5.0	1.6
3	30.0	22.5	7.5	2.4
4	40.0	30.0	10.0	3.2
5	50.0	37.5	12.5	4.0
6	60.0	45.0	15.0	4.8
7	70.0	52.5	17.5	5.6
8	80.0	60.0	20.0	6.4
9	90.0	67.5	22.5	7.2
10	100.0	75.0	25.0	8.0

2C) PCR Thermocycling

Transport all chilled PCR tubes (strips) prepared as above to a place where PCR runs are performed. Programme the Mastercycler (Eppendorf) as described (Tab. 4). After the PCR runs go to section 2D for the detection of amplicons.

Tab. 4: PCR programme for Mastercycler (Eppendorf)

Method	Cycles	Target temperature [°C]	Incubation time [hh:mm:ss]
Initial denaturation	1	95	00:01:00
Cycling	40	95	00:00:05
		55	00:00:05
		72	00:00:25
Cooling	1	10	00:00:10

2D) Detection by Agarose Gel Electrophoresis

After thermocycling, transport the PCR tubes to a place where DNA is handled. Use components of Kit 4A.

! For equipment, consumables and reagents to be supplied by the user (page to 7).

! The DNA staining solution, which is present in the mastermixes during PCR amplification is used for gel electrophoretic visualisation of the amplicon DNA. Make sure that the tubes are kept in the dark until gel electrophoresis. Thaw the DNA size marker (*SM*). The DNA size marker should be kept at +4 to +12°C in the dark for further storage (do not re-freeze).

Protocol:

Prepare the gel following the instructions of the manufacturer or prepare a gel in 1x TAE buffer. Place the gel in a tray, transfer into the chamber and fill with freshly prepared 1x TAE running buffer as instructed by the manufacturer (the gel should be covered with approx. 1cm buffer).

- Per assay analysis, pipette 8µl of the PCR reaction mixture into a sterile 1.5ml polypropylene tube (not supplied) or in a well of a 96 well plate (not supplied) and mix with 2µl of the gel loading solution (*LS*). Mix by pipetting in and out for several times.
- Pipette the mixture (10µl) into an indentation of the gel. Repeat the procedure with the other PCR reaction mixtures, including samples, internal controls positive PCR controls, negative PCR controls and, at the end pipette 5µl of supplied *SM* (DNA size marker; Kit 4B).
- Close the electrophoresis chamber with the cover and run the gel at 300V (using Consort E835 or at 10V/cm interelectrode distance) in the dark.
- Leave the gel running until the blue dye has moved about 2/3 of the way through the gel. At the conditions described this takes about 30 to 45min.
- Remove the gel, place under a UV lamp or on a transilluminator (260 to 310nm wavelength) and photograph/document. Compare appearing bands of samples with the DNA size marker and positive PCR controls P1 and P2. For an example, see Fig. 3 and Fig. 4, pages 29 to 30.
- Make sure that bands appear with the internal controls, IC and IC_W. This is important in cases of negative samples. Bands in internal control runs indicate the absence of PCR inhibitors in the eluates.

2E) Guidance to the Interpretation of PCR Results

A typical image of the results of the analysis of samples is given in Fig. 3. In this case, samples were collected from six patients and proceeded as described in the previous sections.
The controls were as expected, i.e. positive PCR controls P1 and P2 showed bands at the correct position (approx. 450bp, see 'bacterial DNA' in Fig. 3). The internal controls IC1 to IC6 of the samples 1 through 6 and IC_W (right part of Fig. 3) showed clearly visible bands equal in intensity (approx. 200bp), demonstrating the absence of PCR inhibition. The negative PCR control (NC) did not show a signal, so DNA contamination can be excluded. The results from internal controls and negative PCR control indicate that the assay performed correctly. Banding of amplification products from Assay *MA Yeasts* (fungi) appear at a position of approx. 310bp ('fungal DNA' Fig. 4, page 30).
Three of the samples showed signals in the Assay *MA Bac* at the expected gel position (Fig. 3, left side) and thus were positive for bacterial DNA. The other three samples were negative (only showing weak bands at approx. 320bp, indicating traces of human DNA amplified; 'human DNA', Fig. 3). Sequencing analysis of the positive samples, using supplied sequencing primers *SeqGP16* and *SeqGN16* (Identification of Pathogens by Sequencing, pages 30 to 31) indicated the presence of *Staphylococcus epidermidis* (samples no. 1 and 2) and *Escherichia coli* (sample no. 5) in the samples.

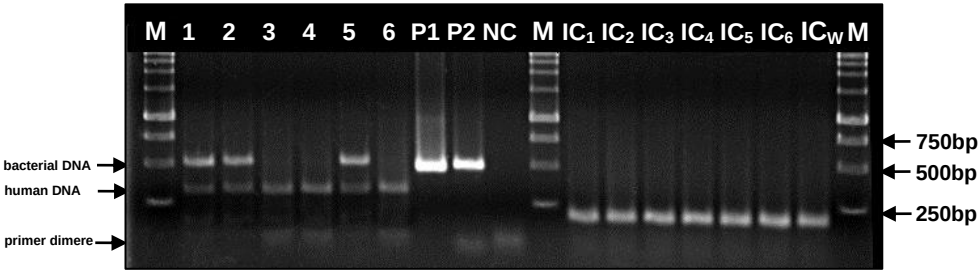


Fig. 3: PCR analysis of eluates from 6 patient samples (1 to 6) using Mastermix Assay Bacteria (*MA Bac*). P1, P2: Positive PCR controls; NC: Negative PCR control; IC1 through IC6: Internal controls with respective eluates from patient sample; IC_W: Internal control with DNA-free water as a reference (banding at approx. 200bp); M: DNA size marker (SM). The weak unspecific signals (left side of the figure; arrow 'human DNA', approx. 320bp) below the specific signals from samples (arrow 'bacterial DNA', approx. 450bp) are the result of the amplification of traces of human DNA, well separated from the specific bands.

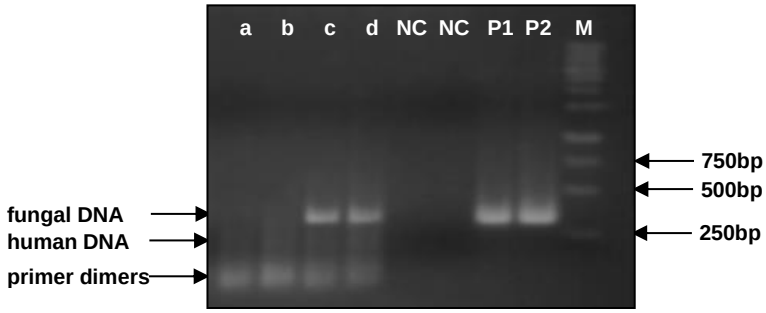


Fig. 4: PCR analysis of eluates from four patient samples (a to d) using Mastermix Assay Yeasts (*MA Yeasts*, fungi). P1, P2: Positive PCR controls; NC: Negative PCR control; M: DNA size marker (SM). Signals at the position 'fungal DNA' (310bp) indicate that samples of patients c and d contain fungal DNA (sequencing result: *Candida albicans*). The weak signals at approximately 250bp (arrow 'human DNA') are the results of the amplification of traces of human DNA.

Identification of Pathogens by Sequencing of Amplicons

Sequencing of amplicons together with BLAST online homology search is used for the identification of pathogens detected by ***SepsiTest™***. Sequence analysis has been validated with ***SepsiTest™***. Online BLAST tools are available, e.g., NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The free online tool, SepsiTest™-BLAST, is a user friendly way of identification of pathogens relying on quality-controlled reference data sets of more than 7,000 sequences from cultured bacteria, yeasts, *Cryptococcus* spp. and *Aspergillus* spp. (http://www.sepsitest-blast.net).

2F) Purification of Amplicons

For sequencing, amplicons need to be purified. Qiagen's QIAquick® PCR Purification Kit (cat. no. 28104) has been validated with ***SepsiTest™***. For this purpose, use the aliquot remaining after analysis of the PCR reaction mixture (approximately **17µl**; 25µl, if using Real-Time PCR; addendum pages 32 to 37) and follow the instructions of the manufacturer of the kit. Elute the purified amplicon from the column (QIAquick®) with **30µl** sterile deionised water.
Continue with the sequencing procedure (section 2G, page 31).

2G) Sequencing

Apply the purified amplicon DNA to a sequencing reaction as advised by the manufacturer of the sequencing system. ***SepsiTest™***. has been validated using Applied Biosystems DNA Analyzer ABI 3730XL® and ABI Prism310® apparatuses together with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). For sequencing, use the sequencing primers (10µM each) supplied with Kit 4. For sequencing of amplicons Assay *MA Bac* (bacteria) use *SeqGP16* and *SeqGN16* in separate reactions, and from Assay *MA Yeasts* (fungi) use *SeqYeast18*. *SeqGP16* and *SeqGN16* are primers binding to regions within the amplicon specific for Gram-positive and Gram-negative bacteria, respectively.

As an example, the following protocol for QIAquick®-purified amplicons using the ABI Prism310® may give satisfying results. Use 2µl of purified DNA for cycle sequencing. Add 4µl Big-Dye® Reaction mix (containing polymerase und nucleotides), 0.5µl sequencing primer *SeqGP16*, *SeqGN16* or *SeqYeast18* (10pmol/µl) and PCR-grade water to fill up to a final volume of 20µl. Incubate in the Eppendorf PCR machine under the following conditions: Initial denaturation at 95°C for 1min; 26 cycles at 95°C for 30s, 55°C for 30s and 60°C for 4min. Apply the sequencing reaction to a CentriSep® column (Princeton Separations, Adelphia, NJ, USA) and spin the column at 750x*g* for 2min. Combine 5µl of the eluate containing the products of the sequencing reaction with 20µl formamide (or TSR reagent containing formamide) and incubate at 95°C for 4min. Apply the reaction mix to the capillary of the ABI Prism310®.

Validate the performance of the used sequencing system. For this, analyse the purified amplicons of the positive PCR controls P1 and P2. Both controls should give readable results.

Alternatively use an overnight sequencing service (e.g., GATC Biotech AG, Konstanz, Germany).

2H) SepsiTest™-BLAST Analysis for Strain Identification

Molzym has developed a free online service (www.sepsitest-blast.net) for the small subunit rRNA genes-based identification of bacteria and fungi. The identification relies on an algorithm for the comparison of input sequences with a reference sequence data library. SepsiTest™-BLAST is characterised by a pool of more than 7,000 quality-controlled complete sequences of the 16S and 18S rRNA genes of only cultured and denominated eubacteria, yeasts, *Cryptococcus spp*. and *Aspergillus spp*. The tool is very simple to use and results are obtained as an output of hits in the order of decreasing sequence identity scores.

Note: Sequence identities ≥97 to <99% should be interpreted as on the genus level, ≥99% as on the species level (Wellinghausen et al. 2009). Sequence identities below 97% are rejected by SepsiTest™-BLAST. This may be the result of reading errors of the sequencing reaction. In such a case it is recommended to inspect the densitogram read-out for overlying sequences indicating the presence of more than one strain in the sample (Fig. 2, page 12). Overlying sequences can be resolved using a specialised tool, RipSeq® (http://www.isentio.com).

Addendum: Real-Time PCR Protocols

In the following, protocols for Real-Time PCR are provided which are based on extensive evaluation to demonstrate their performance.

Please note that ***SepsiTest™*** does not provide a licence for the use of Real-Time PCR (legal aspects, below). In the following, protocols are described for Roche LightCycler® 1.5, 2.0, 96, 480 and Nano Real-Time PCR machines, BioRad Opticon® DNA Engine, ABI 7500 FAST, Stratagene Mx3000P and Qiagen Rotor-Gene. Other instruments may be validated for their use with this kit by the user. At the end, (pages 35 to 37) a guidance to the interpretation of possible results is given.

Patents/Disclaimer

Use of this product is limited to PCR as described in the previous sections (pages 22 to 31). Other applications, in particular Real-Time PCR, for which this product is described below, is covered by patents issued and applicable in certain countries. Because purchase of this product does not include a license to perform any patented application other than covered by patents of Molzym, users of this product may be required to obtain a patent license depending upon the particular application and country in which the product is used. Patents especially to be mentioned are those for Real-Time PCR and the use of intercalating fluorescent dyes and probes: EP 543942, EP 919565, US 5210015, US 5487972, US 5804375, US 6214979, EP 512 334, US 5994056, US 6171785, US 5538848, US 5723591, US 5952202, US 5876930, US 6030787, US 6258569, US 6821727.

General requirements

Please take notice of the general requirements for the performance of PCR (part 2, pages 23 to 27).

! Calibrate your Real-Time PCR machine using the Mastermix *MA Bac* (Kit 3). Perform Real-Time PCR runs with supplied Standard DNA P1 and P2 according to the protocol described on pages 19 to 27. The specific thermocycling conditions are described on pages 33 to 35. For the preparation of mastermixes follow the instructions (part 2, 2A to 2B, pages 24 to 27). Use the DNA-free water (H_2O) supplied with kit 3 for the calibrations. Both positive PCR controls, P1 and P2, must show a target-specific peak.

! For equipment, consumables and reagents to be supplied by the user see page 7. In addition, the following items are needed to perform Real-Time PCR:

- 1x Real-Time PCR machine (above). Other instruments have to be validated for their use.
- PCR tubes e.g., glass capillaries (20µl) for Lightcycler®1.5 (25µl final volume per assay) or PCR strips (8x 0.2ml) for other systems; e.g., Biozym Flat Optical 8-Cap Strip (order no. 712109)

! To avoid contamination it is important that the setup of *MA Bac* and *MA Yeasts* is performed in a lab separated from the setup of *MA IC* (as well as from DNA extraction and PCR amplifications).

Caution: Wear sterile protective gloves, sterile disposable sleeve covers, a disposable lab coat, protective goggles, a bouffant cover and disposable overshoes during the setup of mastermixes. Work in a PCR workstation irradiated with UV before starting according to the instruction manual of the manufacturer. Follow the instructions of the manufacturer for maintenance of the PCR workstation. Do not work under UV irradiation.

Real-Time PCR Thermocycling and Detection by Melting Curve Analysis

A melting curve analysis has always to be performed in order to discriminate possible primer dimer formation from true pathogen signals. See examples on pages 35 to 37. For sequencing of amplicons see part 2, section 2E to 2H, pages 30 to 31.

I) Roche LightCycler® 1.5 and 2.0 (25µl final volume per assay)

Transport filled capillaries to a place where PCR runs are performed and programme the Real-Time PCR machine as described below. Set the appropriate channel to SYBR® Green 1 detection.

Method	Cycles	Analysis Mode	Target Temperature [°C]	Incubation time [hh:mm:ss]	Ramp rate [°C/s]	Acquisition [per °C]	Acquisiton Mode
Initial denaturation	1	None	95	00:01:00	20.00	-	NONE
Cycling	40	Quantification	95	00:00:05	20.00	-	NONE
			55	00:00:05	20.00	-	NONE
			72	00:00:25	20.00	-	SINGLE
Melting	1	Melting Curve	95	00:00:00	20.00	-	NONE
			65	00:00:15	20.00	-	NONE
			95	00:00:00	0.05	-	CONT
Cooling	1	None	40	00:00:05	20.00	-	NONE

II) Roche LightCycler® 96

Method	Cycles	Analysis Mode	Target Temperature [°C]	Incubation time [hh:mm:ss]	Ramp rate [°C/s]	Acquisition [per °C]	Acquisiton Mode
Initial denaturation	1	None	95	00:01:00	4.40	-	None
Cycling	40	Quantification	95	00:00:05	4.40	-	None
			55	00:00:10	2.20	-	None
			72	00:00:25	4.40	-	Single
Melting	1	Melting Curve	95	00:00:01	4.40	-	None
			70	00:00:01	2.20	-	None
			95	-	0.04	25	Continuous
Cooling	1	None	40	00:00:10	-	-	-

III) Roche LightCycler® 480

Method	Cycles	Analysis Mode	Target Temperature [°C]	Incubation time [hh:mm:ss]	Ramp rate [°C/s]	Acquisition [per °C]	Acquisiton Mode
Initial denaturation	1	None	95	00:01:00	4.40	-	None
Cycling	40	Quantification	95	00:00:05	4.40	-	None
			55	00:00:10	2.20	-	None
			72	00:00:25	4.40	-	Single
Melting	1	Melting Curve	95	00:00:01	4.40	-	None
			70	00:00:01	2.20	-	None
			95	-	0.11	5	Continuous
Cooling	1	None	40	00:00:10	-	-	-

IV) Roche LightCycler® Nano

Set the appropriate channel to SYBR® Green I detection.

Method	Cycles	Programs	Target Temperature [°C]	Incubation time [hh:mm:ss]	Ramp rate [°C/s]	Acquisition Mode
Initial denaturation	1	Hold	95	00:01:00	5.00	
Cycling	40	Quantification	95	00:00:05	2.00	
			55	00:00:05	2.00	
			72	00:00:25	2.00	✓ Acquire
Melting	1	Melting	60	00:00:20	4.00	
			95	00:00:20	0.1	
Cooling	1	Hold	40	00:00:05	5.00	

V) BioRad DNA Engine Opticon®

Method	Cycles	Target Temperature [°C]	Incubation time [hh:mm:ss]	Ramp rate [°C/s]	Acquisition Mode
Initial denaturation	1	95	00:01:00		
Cycling	40	95	00:00:05		
		55	00:00:05		
		72	00:00:25		Reading point after 72°C step
Method	**Cycles**	**Melting Curve**			
Melting Curve	1	from 70°C to 95°C	Read every 0.2°C, hold for 1s between reads		

VI) ABI 7500 Fast®

Method	Cycles	Target Temperature [°C]	Incubation time [hh:mm:ss]	Ramp rate [°C/s]	Acquisition Mode
Initial denaturation	1	95	00:01:00		
Cycling	40	95	00:00:05		
		55	00:00:10		
		72	00:00:25		on
Melting Curve	1	95	00:00:15		
		70	00:01:00		
		95		0.2	
		95	00:00:15		
Cooling	1	60	00:00:15	100%	

VII) Stratagene Mx3000P®

Method	Cycles	Target Temperature [°C]	Incubation time [hh:mm:ss]	Amplification averaging point	Dissociation averaging points	Dissociation point separation
Initial denaturation	1	95	00:01:00			
Cycling	40	95	00:00:15			
		55	00:00:15			
		72 (reading point)	00:00:30			
Melting Curve	1	95	00:01:00			
		55	00:00:30			
		95		3	3	0.5°C

VIII) Qiagen Rotor-Gene®

To program a new run for melting curve analyses select: Three steps with Melt.

Amplification				
Method	**Cycles**	**Target Temperature [°C]**	**Incubation time [hh:mm:ss]**	**Acquisition Mode**
Hold	1	95	00:01:00	
Cycling	40	95	00:00:05	
		55	00:00:10	
		72	00:00:25	Acquiring to cycle A; Acquiring channel A

Melting				
Method	**Ramp Parameters**			**Acquire**
Melt	from	70	degrees	Melt A; on Green
	to	95	degrees	
	Rising by	0.2	degree(s) each step	
	Wait for	90	seconds of pre-melt conditioning on first step	
	Wait for	1	seconds for each step afterwards	
	Grain-Optimisation			
	☐ Optimise gain before melt on all tubes The gain giving the highest fluorescence less than will be selected			95

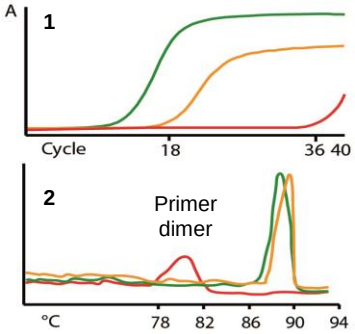
Guidance to the Interpretation of Real-Time PCR Results

This kit supplies assays for the detection of eubacteria and fungi, respectively. The assays are based on the amplification of the 16S and 18S rRNA genes, respectively. The advantage of this approach is that, in principle, all microorganisms are detected irrespective of the taxonomic status of the strain. The drawback, on the other side, of such a universal system is that the assays are sensitive to contamination by exogenous DNA introduced to the assays by aerosols or direct carryover between samples. Hence, the results of Real-Time PCR runs can lead to diverse appearances. In the following, besides true results, a selection of typical false results are presented and discussed. In this illustration it is understood that the positive controls indicate full functioning of the assay.

Examples (A to G) are shown in a schematic modus of Real-Time PCRs (upper image), indicating relevant crossing points (C(t)) of the reactions (numbers as cycles), and of melting curves (lower image). Absolute and relative T_m values can vary among different Real-Time PCR systems. On the right hand side the interpretation of the results is given in tables and text. The colour code in the table corresponds to the curves in the images.

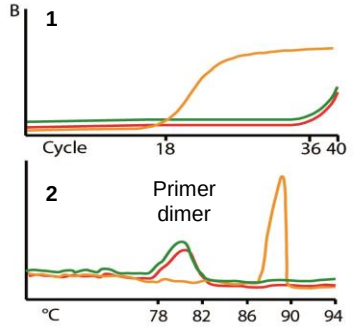
Legend:

Sample: Mastermix Assay Bacteria (*MA Bac*) or Mastermix Assay Yeasts (*MA Yeasts*) – green curve; **IC**: Mastermix Assay Internal Control (*MA IC*) – orange curve; **NC**: Negative PCR control – red curve; **Pathogen present?**: **+** means a true positive result, **-** means a true negative result, **?** means that the result is unclear. **Figures: 1** Amplification curves; **2** Melting curve analysis.



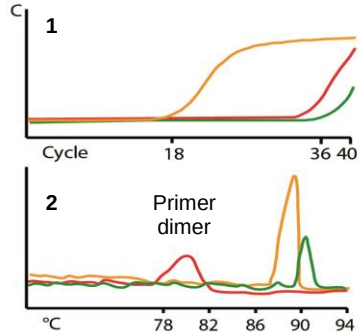
Sample	IC	NC	Pathogen present?
+	+	–	+

True positive result
The internal control appears at the expected value (here C(t) 18). The sample is positive in the melting curve analysis and the negative control does not show a signal (besides primer dimers).



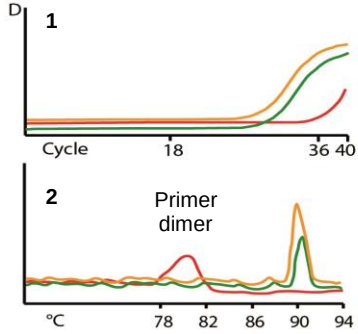
Sample	IC	NC	Pathogen present?
–	+	–	–

True negative result
The internal control is at the expected value. The sample and negative control lack a peak in the melting curve analysis (only primer dimers). Hence, pathogens are not present or below the detection limit.



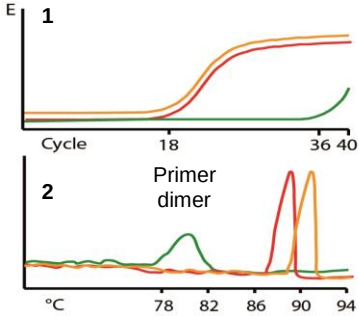
Sample	IC	NC	Pathogen present?
+	+	–	+

True positive result
The internal control is at the regular value. The sample shows a peak in the melting curve, while the negative control does not. The high C(t) value with the sample indicates a low pathogen DNA load in the sample.



Sample	IC	NC	Pathogen present?
+	–/+	–	+

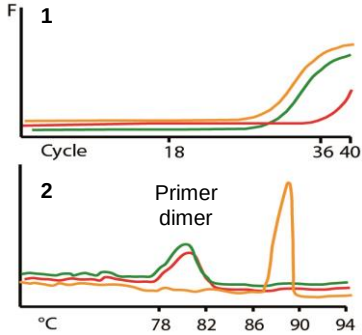
True positive result
The internal control shows a shift to a higher C(t) value as a result of PCR inhibition. Despite this, a peak in the melting curve with the sample occurs, indicating the presence of pathogen DNA.



Sample	IC	NC	Pathogen present?
–	+	+	–

True negative result

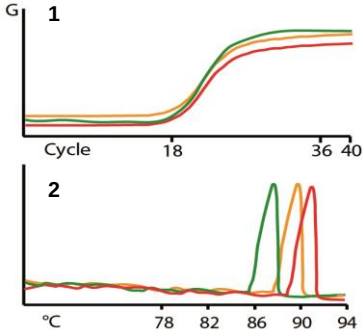
The internal control is regular and the sample lacks a signal. Despite a signal in the negative control, which may be the result of contamination by handling, pathogens are probably not present in the blood sample or below the detection limit. However, for verification, the PCR should be repeated.



Sample	IC	NC	Pathogen present?
–	–/+	–	?

False negative result

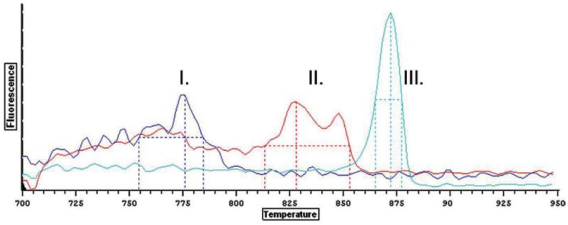
The shift of the C(t) of the internal control indicates PCR inhibition. The sample lacks a signal, either because of inhibition or a very low pathogen load. In such a case, the extraction has to be repeated.



Sample	IC	NC	Pathogen present?
+	+	+	?

False positive result

The negative control shows a strong signal, indicating contamination. It is not clear whether the signal of the sample comes from contamination or from a pathogen in the sample. The PCR should be repeated with more care to avoid contamination.



Melting curve analysis (BioRad DNA Engine Opticon®) of a negative PCR control (I.), an eluate of a negative sample showing peaks of amplified traces of human DNA (II.) and a positive PCR control showing a specific peak (III.). Reproduced from Mühl et al. (2008).

Supplementary Information

Troubleshooting

This guide may help solve problems that may arise. For further support:
Phone: +49(0)421 69 61 62 0; E-Mail: support@molzym.com

Observation	Possible cause	Comments/suggestions
Strong human DNA background in gel electro-phoresis or Real-Time PCR	• Buffer *CM* not added • Buffer *DB1* not added • *MolDNase B* not added	Eluates usually contain traces of human DNA co-eluted with bacterial/fungal DNA (Fig. 3 and Fig. 4 pages 29 to 30). If the extraction has not been performed according to the protocol, increased amounts of human DNA can be the result, which negatively influences the PCR reaction. Ensure that buffer *CM* has been added to lyse human cells. Accordingly, addition of buffer *DB1* and *MolDNase B* is obligate. Keep the *MolDNase B* vial chilled, because warming may reduce enzyme activity and hence increase human DNA background. It is important that solutions are thoroughly mixed after addition of buffers. Follow instructions for vortexing.
No pathogen DNA detectable (spiking test with negative blood)	• Insufficient lysis • PCR inhibition	Make sure that *BugLysis* and ß-mercaptoethanol have been added. Ensure that the *Proteinase K* treatment has been done. Run the internal control assay (*MA IC*) for testing for potential PCR inhibition.
	• Insufficient homogenisation • Pathogen titre too low	If the pellet at steps 4 and 6 (page 20) is not homogenised, pathogen cells may be included in the debris and not reached by lytic enzymes. Follow the instructions. Check the titre of the pathogen by plating and increase the titre for inoculation.
	• Loss of nucleic acids during purification	Ensure that buffer *AB* has been added to and mixed with the lysate (step 10, page 21). Accordingly, make sure that the column has been washed with buffer WB (step 12, page 21).
	• Wrong elution conditions	Make sure to elute with supplied heated buffer ES (70°C; step 15, page 21). This increases the DNA yield significantly.
False positive result (signal in negative PCR control)	• Cross contamination • Contamination during handling	Principally, work under UV-irradiated workstations. Avoid the generation of aerosols by careful pipetting. Open vials and tubes only shortly for pipetting and close again immediately thereafter. Frequently change gloves. UV-irradiate the workstation at the end of handling a series of samples. Prepare mastermixes and handle DNA samples under different UV workstations (page 16). Use DNA-free pipette tips and other plastics only as recommended (page 7)
False negative result (no signal in Assay *MA IC* [internal control])	• PCR inhibitors co-eluted	Check whether *Proteinase K* treatment has been performed during DNA preparation. Make sure that all washing steps of the procedure have been followed. Optionally, after buffer *WS* washing (step 13, page 21), centrifuge for another 1min to avoid ethanol carryover to the eluate.

References

DIN 58967-60 (**Oktober 2001**) Serologische und molekularbiologische Diagnostik von Infektions– und Immunkrankheiten. Teil 60: Polymerase-Kettenreaktion (PCR).

Espy M.J., Uhl J.R., Sloan L.M., Buckwalter S.P., Jones M.F., Vetter E.A., Yao J.D.C., Wengenack N.L., Rosenblatt J.E., Cockerill F.R. and Smith T.F. (**2006**) Real-Time PCR in clinical microbiology: applications for routine laboratory testing. *Clin. Microbiol. Rev*. 19: 165-256

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