SepsiTest-UMD

REF **U-010-024 / U-010-048**

Sample Pre-treatment, Bacterial / Fungal DNA Isolation and PCR Analysis

Internal extraction control assay, positive PCR control and sequencing primers included

Body fluids

(*ascites, BAL, blood, blood culture, CSF, plasma, pleural fluid, pus, synovial fluid*)

Swabs

(mouth, nasopharynx, wounds, bones)

Tissues

(*abscesses*, *biopsies, heart valves, prostheses*)

– For *in vitro* diagnostic use –

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Version 04

Date of first release: 05/2017
Last update: 05/2026

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Kit Information

Kit Content – *SepsiTest-UMD*

DNA Isolation	Σ/24 (U-010-024)	Σ/48 (U-010-048)	Single use
Kit 1 - Buffers & Consumables (store at +18 to +25°C)			
A) Extraction Buffers, in DNA-free bags			
CM	6x 1.0 ml	12x 1.0 ml	No
DB1	6x 1.0 ml	12x 1.0 ml	No
RS	6x [4x 1.0 ml]	12x [4x 1.0 ml]	(single use symbol)
RL	6x 0.32 ml	12x 0.32 ml	No
RP	6x 0.6 ml	12x 0.6 ml	No
CS	6x 1.0 ml	12x 1.0 ml	No
AB	6x 1.0 ml	12x 1.0 ml	No
WB	6x 1.6 ml	12x 1.6 ml	No
WS	6x 1.6 ml	12x 1.6 ml	No
ES	6x 0.4 ml	12x 0.4 ml	No
B) Consumables, in DNA-free bags			
ST - Sample tubes, 2.0 ml	6x 4	12x 4	(single use symbol)
C) Consumables, in DNA-free bags			
SC – Spin columns in 2.0 ml *Collection tubes*	6x 4	12x 4	(single use symbol)
CT - Collection tubes, 2.0 ml	6x 8	12x 8	(single use symbol)
ET – Elution tubes, 1.5 ml	6x 4	12x 4	(single use symbol)
D) Tissue Pre-treatment Buffers, in white boxes			
TSB	1x 25 ml	2x [1x 25 ml]	No
PKB	4x 1.2 ml	2x [4x 1.2 ml]	No
E) Sample Dilution Buffer, in white boxes			
SU	2x [12x 1.0 ml]	4x [12x 1.0 ml]	(single use symbol)
DNA Isolation	**Σ/24 (U-010-024)**	**Σ/48 (U-010-048)**	**Single use**
Kit 2 – Enzymes & Reagents (store at -15 to -25°C), in white boxes			
Enzyme K, solution	2x [3x 0.08 ml]	4x [3x 0.08 ml]	No
MolDNase B, solution	2x [3x 0.04 ml]	4x [3x 0.04 ml]	No
BugLysis, solution	2x [3x 0.08 ml]	4x [3x 0.08 ml]	No
ß-mercaptoethanol, solution	2x [3x 0.08 ml]	4x [3x 0.08 ml]	No
Proteinase K, solution	2x [3x 0.08 ml]	4x [3x 0.08 ml]	No
Internal Extraction Control Unit (store at -15 to -25°C), in bags (in Kit 2)			
Control DNA	2x [3x 0.01 ml]	4x [3x 0.01 ml]	No

Continued on next page

PCR Detection and Identification	Σ 24 (U-010-024)	Σ 48 (U-010-048)	Single use
Kit 3 - PCR Reagents (store at -15 to -25°C), in white boxes			
MA Bac, Mastermix Assay Bacteria, 2.5x conc.	2x 0.30 ml	2x [2x 0.30 ml]	No
MA Yeasts, Mastermix Assay Yeasts, 2.5x conc.	2x 0.30 ml	2x [2x 0.30 ml]	No
MA Control, Mastermix Assay Control, 2.5x conc.	1x 0.36 ml	2x [1x 0.36 ml]	No
MolTaq 16S/18S	3x 0.05 ml	2x [3x 0.05 ml]	No
H_2O - DNA-free PCR-grade water	3x 0.75 ml	2x [3x 0.75 ml]	No
DS - DNA staining solution, 10x conc.	2x 0.30 ml	2x [2x 0.30 ml]	No
Kit 4 - PCR Controls & Detection Reagents (store at -15 to -25°C)			
A) Detection Reagents (white box)			
LS - Gel loading solution, 6x conc.	1x 0.4 ml	1x 0.4 ml	No
SM - DNA size marker	1x 0.24 ml	1x 0.24 ml	No
SeqGP16 - Sequencing primer (bacteria)	1x 0.1 ml	2x 0.1 ml	No
SeqGN16 - Sequencing primer (bacteria)	1x 0.1 ml	2x 0.1 ml	No
SeqYeast18 - Sequencing primer (fungi)	1x 0.1 ml	2x 0.1 ml	No
B) Positive PCR Control (bag)			
DNA Standard P1, for PCR positive control runs	1x 0.3 ml	1x 0.3 ml	No
DNA dilution buffer (for P1)	1x 25 ml	2x 25 ml	No
Consumables PCR Detection & Identification (store at +18 to +25°C), in Kit 1			
MT - Mastermix tubes, 1.5 ml (for Kit 3)	2x 50	3x 50	(Do not re-use)

Manuals (in Kit 1)	Σ 24 (U-010-024)	Σ 48 (U-010-048)	Single use
Manual	1x	1x	No
Short manual sheets	4x	4x	No

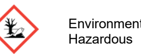
Symbols

Symbols used in labelling and in section 'Hazard and Precautionary Statements' (pages 12 to 13).

Informative symbols

Symbol	Meaning	Symbol	Meaning	Symbol	Meaning
LOT	Batch code	Cont.	Content of the package		Keep away from sunlight
REF	Catalogue number		Do not re-use		Manufacturer
	Caution	CE	European Conformity		Temperature limitation (store at)
	Consult instructions for use	IVD	*In vitro* diagnostic medical device		Use by
Σ	Contains sufficient for <n> tests	CH REP	Swiss authorised representative		

Hazard pictograms

	Flammable		Toxicity		Health hazard
	Corrosive		Irritant		Environmentally Hazardous

Storage and Stability

Guarantee for **full performance** of reagents and buffers is given through the **expiration date** printed on the label at the outer box, if the **packed material is undamaged** upon arrival and the reagents are unopened.

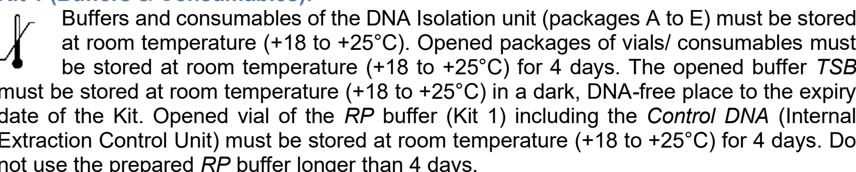
Please ensure upon arrival of the kits that the **frozen goods (Kit 2, Kit 3 and Kit 4) are still frozen or contain dry ice**.

Guarantee for full performance of ***SepsiTest-UMD*** as specified in this manual is only valid if storage conditions are followed (Table 1).

Once opened, the vials have to be used as specified by the protocol.

The DNA Isolation units (Kit 1 and 2) and the Internal Extraction Control Unit (Kit 2), are provided in vials containing reagents for the extraction of 4 samples. This minimises the risk of carry-over contamination.

Kit 1 (Buffers & Consumables):



Buffers and consumables of the DNA Isolation unit (packages A to E) must be stored at room temperature (+18 to +25°C). Opened packages of vials/ consumables must be stored at room temperature (+18 to +25°C) for 4 days. The opened buffer *TSB* must be stored at room temperature (+18 to +25°C) in a dark, DNA-free place to the expiry date of the Kit. Opened vial of the *RP* buffer (Kit 1) including the *Control DNA* (Internal Extraction Control Unit) must be stored at room temperature (+18 to +25°C) for 4 days. Do not use the prepared *RP* buffer longer than 4 days.

Buffers and consumables of the DNA Isolation unit (packages A to E) must be kept away from sunlight. Opened buffers of Kit 1A must be kept away from sunlight.

Kit 2 (Enzymes & Reagents):

Please take care that the vials of the DNA Isolation unit have to be stored at -15 to -25°C upon delivery.

Internal Extraction Control Unit (Kit 2):

Please take care that the vials of the Internal Extraction Control Unit have to be stored at -15 to -25°C upon delivery.

Kits 3 (PCR Reagents) and 4A (Detection Reagents):

Please take care that the vials of the PCR Detection unit (Kits 3 and 4A) must be stored at -15 to -25°C upon delivery. ***Do not freeze again*** vial *DS*, *SM* and *LS*, and store them at +4 to +12°C for further use. After use, the mastermixes and H_2O must be stored in the refrigerator (+4 to +12°C) for further use at the same day but must be replaced to -15 to -25°C for longer storage.

It is important to note that the DNA staining solution (*DS*, Kit 3) and the DNA size marker (*SM*, Kit 4A) are sensitive to light and must be stored dark during handling and storage.

Kit 4B (Positive PCR Control):

Please take care that the components of the PCR Detection unit (Kit 4B) must be stored at -15 to -25°C upon delivery. The reagents of Kit 4B must be stored at +4 to +12°C after the first use.

Table 1: Storage of the *SepsiTest-UMD* components (*exp. date: expiry date of the kit).

Components	**Storage** Temperature	**Working** Temperature	**Storage & Stabilitiy after the** Temperature	Days (dark)*
Kit 1 - Buffers & Consumables:				
CM, DB1, RS, RL, RP, CS, AB, WS, ES				4
Consumables (*ST, SC, CT, ET*)			+18 to + 25°C	4
TSB	+18 to + 25°C	+18 to + 25°C		exp. date
PKB				4
SU			single-use, no further storage	
Kit 2 - Enzymes & Reagents:				
Enzyme K, MolDNase B, BugLysis, ß-mercaptoethanol, Proteinase K	-15 to -25°C	-15 to -25°C	-15 to -25°C	exp. date
Internal Extraction Control Unit (in Kit 2):				
Control DNA	-15 to -25°C	+18 to + 25°C	+18 to + 25°C in buffer RP	4
Kit 3 - PCR Reagents:				
MA Bac, MA Yeasts, MA Control		+18 to + 25°C	+4 to + 12°C -15 to -25°C	1 (thereafter freeze) exp. date
MolTaq 16S/18S	-15 to -25°C	-15 to -25°C	-15 to -25°C	exp. date
H2O		+18 to + 25°C	+4 to + 12°C -15 to -25°C	1 (thereafter freeze) exp. date
DS (*DNA Staining Solution*)		+18 to + 25°C	+4 to + 12°C	exp. date
Kit 4A - Detection Reagents:				
LS (Gel loading solution)			+4 to + 12°C	exp. date
SM (DNA size marker)	-15 to -25°C	+18 to + 25°C	+4 to + 12°C	exp. date
SeqGP16, SeqGN16, SeqYeast18			-15 to -25°C	exp. date
Kit 4B - Positive PCR Control:				
P1 DNA Standard	-15 to -25°C			exp. date
P2 (prepare freshly for the PCR), 1:500 dilution of P1	none	+18 to + 25°C	+4 to + 12°C	1
DNA Dilution Buffer	-15 to -25°C			exp. date
Consumables PCR Detection & Identification, in Kit 1:				
MT - Mastermix tubes, 1.5 ml (for Kit 3)	+18 to + 25°C	+18 to + 25°C	+18 to + 25°C	exp. date

Intended Use and Indication

SepsiTest-UMD is a kit for the detection of microbial DNA (bacterial/fungal). It is a set of reagents to detect the presence and identify bacteria and fungi in primary-sterile specimens. ***SepsiTest-UMD*** is intended for body fluids, swabs and for tissues (validated specimens see Table 3). The kit is for laboratory use (professional users).

SepsiTest-UMD is a kit for sample analysis of patients with suspected bacterial or fungal infection.

Contraindication

SepsiTest-UMD reagent kit is not intended to be used as in vitro diagnostic test for the detection and identification of any specific pathogen. The results of ***SepsiTest-UMD*** are not used as the sole basis for diagnosis, treatment, or other patient management decisions. ***SepsiTest-UMD*** is not indicated for pathogens with safety level S3 and S4. An exemplary selection is listed in Table 2.

Table 2: Contraindication of ***SepsiTest-UMD*** for pathogens with safety level S3 and S4 (exemplary selection).

Bacillus cereus biovar anthracis	*Mycobacterium microti*
Brucella abortus (B. melitensis biovar abortus)	*Mycobacterium pinnipedii*
Brucella canis (B. melitensis biovar canis)	*Mycobacterium tuberculosis (Mycobacterium tuberculosis subsp. tuberculosis)*
Brucella inopinata	*Mycobacterium ulcerans*
Brucella melitensis (B. melitensis biovar melitensis)	*Orientia tsutsugamushi (Rickettsia tsutsugamushi)*
Brucella neotomae (B. melitensis biovar neotomae)	*Rickettsia africae*
Brucella ovis (B. melitensis biovar ovis)	*Rickettsia akari*
Brucella suis (B. melitensis biovar suis)	*Rickettsia australis*
Burkholderia mallei (Pseudomonas mallei)	*Rickettsia conorii*
Burkholderia pseudomallei (Pseudomonas pseudomallei)	*Rickettsia heilongjiangensis*
Chlamydia psittaci (Chlamydophila psittaci)	*Rickettsia japonica*
Coxiella burnetii	*Rickettsia prowazekii*
Escherichia coli (enterohemorrhagic (EHEC) Strains O157:H7 or O103)	*Rickettsia rickettsii*
Francisella tularensis subsp. tularensis	*Rickettsia sibirica*
Mycobacterium africanum	*Rickettsia typhi*
Mycobacterium bovis	*Salmonella Typhi*
Mycobacterium caprae (Mycobacterium tuberculosis subsp. caprae)	*Shigella dysenteriae*
Mycobacterium leprae	*Yersinia pestis*

Product Use Limitations

Usage of ***SepsiTest-UMD*** reagents for clinical diagnostic tests requires validation of the in vitro diagnostic test procedure!

Whole blood samples must be collected and stabilized using either EDTA or citrate.

Sequencing results must be validated by a clinician to exclude false positive results originating from contaminations or clinically not relevant microorganisms.

SepsiTest-UMD is not intended for frozen and thawed specimen materials. Not for other specimens than mentioned in Table 3.

Bacterial and fungal cells must be intact for reliable results. This requires specimens not to be stored in solutions, which induce cell lysis.

Transport media including charcoal medium and Amies hold a risk of inhibiting the amplification and should be avoided. Test procedures must always be run including the extraction control and control assay provided with this kit.

Table 3: ***SepsiTest-UMD*** is validated with the following specimens.

Fluid samples	Swabs	Tissue samples
Ascites	Bones	Abscesses
BAL (bronchoalveolar lavage)	Mouth	Biopsies
Blood (EDTA or citrate stabilized)	Nasopharynx	Heart Valves
Blood culture	Wounds	Prostheses
CSF (cerebrospinal fluid)		
Plasma		
Pleural fluid		
Pus		
Synovial fluid		

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-UMD***.

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 preparation:

- 1x thermomixer (2.0 ml tubes), e.g., Eppendorf comfort, Eppendorf, Germany
- 1x cooling rack for 1.5 ml 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 Class II biological safety cabinet
- Sample positive control (run control):
  e.g., BioBall® MultiShot 550 KBE, bioMérieux, Germany
  - BioBall® MultiShot *Candida albicans* NCPF 3179 (56003)
  - BioBall® MultiShot *Escherichia coli* NCTC 12923 (56006)
  - BioBall® MultiShot *Staphylococcus aureus* NCTC 10788 (56009)
- A set of precision pipettes: up to 10 µl, up to 20 µl, up to100 µl, up to 200 µl and up to 1000 µl, e.g., Eppendorf, Germany
- Sample racks

Only tissue protocol

- Sterile forceps (only tissue protocol)
- Sterile support, e.g., Petri dish (only tissue protocol)
- Sterile scalpel or sterile preparation scissors (only tissue protocol)

PCR amplification:

- 1x UV workstation, e.g., GuardOne® Werkbank, Starlab, Germany
- 1x low speed mini-centrifuge (≤2000x*g*) e.g., VWR, Darmstadt, Germany
- 1x vortexer, e.g., VWR, Darmstadt, Germany
- 1x cooling rack for 1.5 ml tubes (-15 to -25°C)
- 3x cooling racks for 0.2 ml PCR tubes (-15 to -25°C)
- PCR cycler, e.g., Mastercycler®, Eppendorf, Germany); other cyclers have to be validated by using positive PCR controls P1 and P2 according to the instructions (pages 34 to 38)

  Optional: Real-Time PCR instruments are validated (page 44 to 48)
- 2-3x sets of precision pipettes: up to 10 µl, up to 20 µl, up to100 µl, up to 200 µl and up to 1000 µl, 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 34 cm, 1.5 l buffer volume capacity)
- Running buffer TAE (50x concentrated), e.g., Biozym, Germany
- An electrophoresis chamber with the following characters: 15 x 34 cm, buffer volume capacity: 1.5 litres
- An electrophoresis power supply (300 V, 500 mA), e.g., Consort E835, Sigma-Aldrich, USA
- A gel documentation system, e.g., system from Herolab, Germany

- A set of precision pipettes: up to 10 µl, up to 20 µl, up to100 µl, up to 200 µl and up to 1000 µl, e.g., Eppendorf, Germany

Sequencing:

- A DNA sequencing apparatus, e.g., DNA Analyzer ABI 3730XL®, ABI Prism310®
- Purification of amplicons, QIAquick® PCR Purification Kit, Qiagen (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., Eurofins Genomics, Germany)
- Column for removal of dye terminators prior to sequencing (use of your internal validated material)
- A set of precision pipettes: up to 10 µl, up to 20 µl, up to100 µl, up to 200 µl and up to 1000 µl, e.g., Eppendorf, Germany

Plastic Consumables and reagents:

- Pipette tips (with aerosol filter), Biosphere® plus, Sarstedt, Germany
  - 10 µl type Eppendorf (70.3010.255)
  - 100 µl type Eppendorf (70.3030.255)
  - 300 µl type Eppendorf (70.3040.255)
  - 1000 µl type Eppendorf (70.3050.255)
- 1.5 ml micro tubes, e.g., Biosphere® plus, Sarstedt, Germany (72.706.200)
  - For the preparation of the positive PCR control P2
  - For gel electrophoresis
- PCR tubes, e.g., PCR strip of 4, 200 µl, Biosphere® plus, Sarstedt, Germany (72.990)
- DNA decontamination, e.g., DNA/RNA-ExitusPlus™, AppliChem, Germany (A7089,0100)
- Surface decontamination, e.g., Meliseptol® New Formula (rapid disinfectant, ethanol containing), B. Braun, Germany
- Sterile disposables
  - Lab coat, e.g., VWR, Germany
  - Gloves, e.g., Kimberly-Clark, Germany
  - Sleeves, e.g., Cardinal Health, Ireland
  - Bouffant Covers, e.g., VWR, Germany
  - Hygiene mask, 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.

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

Buffers *CM* and *CS* contain guanidine 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 Class II biological safety cabinet 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 24 to 29) according to the national directive of the health organisation (e.g., in Germany: Vollzugshilfe zur Entsorgung von Abfällen aus Einrichtungen des Gesundheitsdienstes, 2021).
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.

Hazard and Precautionary Statements

Buffer *CM*

Contains guanidine hydrochloride (>10 %):
Acute toxicity (oral) and irritating (eyes and skin).

Warning

Hazard and precautionary statements*(page 13):
H302-H315-H319; P301+P312-P302+P352-P305+P351+P338

ß-mercaptoethanol

Contains 2-mercaptoethanol (100 %, CAS no. 60-24-2):
Acute toxicity (oral, inhalation, skin), irritation (skin), eye damage, skin sensitization, reproductive toxicity, specific target organ toxicity and hazardous to aquatic environment (acute and chronic).

Danger

Hazard and precautionary statements*(page 13):
H301+H331-H310-H315-H317-H318-H361d-H373-H410;
P273-P280-P301+P310-P302+P352+P310-P304+P340+P310-P305+P351+P338

Proteinase K / Enzyme K

Contains *Proteinase K* (≥ 1 %):
Respiratory sensitization and skin sensitization.

Danger

Hazard and precautionary statements*(page 13):
H317-H334; P280-P302+P352-P333+P313-P363

Buffer *RP* / Buffer *PKB*

Contains sodium dodecyl sulfate (< 10 %):
Acute toxicity (oral, inhalation), irritation (skin and eye).

Warning

Hazard and precautionary statements*:
H302-H315-H319-H332; P280-P301+P312-P304+P340+P312-P305+P351+P338

Buffer *CS*

Contains guanidinium thiocyanate (> 10 %): **Acute toxicity (oral, skin), skin sensitization, eye damage and hazardous to aquatic environment (chronic).**

Danger

Hazard and precautionary statements*:
H302-H312-H314-H318-H412-EUH032; P280-P303+P361+P353-P305+P351+P338-P310-P362+P364

Buffer *AB* / Buffer *WB*

Contains 2-propanol (*AB* > 40 % and *WB* ≥ 40 %): **Flammable liquids and irritating (eyes)**.

Danger

Hazard and precautionary statements*:
H225-H319-H336; P210-P233-P305+P351+P338

Buffer *WS*

Contains ethanol (> 50 %): **Flammable liquids and irritating (eyes)**.

Danger

Hazard and precautionary statements*:
H225-H319; P210-P233-P305+P351+P338

Emergency information (24-hours service)

For emergency medical information, please contact the regional poison centre in your country.

* **H225:** Highly flammable liquid and vapour; **H302:** Harmful if swallowed; **H310:** Fatal in contact with skin; **H312:** Harmful in contact with skin; **H314:** Causes severe skin burns and eye damage; **H315:** Causes skin irritation; **H317:** May cause an allergic skin reaction; **H318:** Causes serious eye damage; **H319:** Causes serious eye irritation; **H332:** Harmful if inhaled; **H334:** May cause allergy or asthma symptoms or breathing difficulties if inhaled; **H336:** May cause drowsiness or dizziness; **H361d:** Suspected of damaging the unborn child; **H373:** May cause damage to organs (liver, heart) through prolonged or repeated exposure if swallowed; **H301+H331:** Toxic if swallowed or if inhaled; **H410:** Very toxic to aquatic life with long lasting effects; **H412**: Harmful to aquatic life with long lasting effects; **EUH032**: Contact with acids liberates very toxic gas.
P210: Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking.; **P233:** Keep container tightly closed; **P273:** Avoid release to the environment; **P280:** Wear protective gloves/protective clothing/eye protection/face protection; **P310:** Immediately call a POISON CENTER/doctor; **P363:** Wash contaminated clothing before reuse; **P301+P310:** IF SWALLOWED: Immediately call a POISON CENTER or doctor; **P301+P312:** IF SWALLOWED: Call a POISON CENTER/ doctor if you feel unwell; **P302+P352:** IF ON SKIN: Wash with plenty of water; **P302+P352+P310:** IF ON SKIN: Wash with plenty of water. Immediately call a POISON CENTER/doctor; **P303+P361+P353:** IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water [or shower]; **P304+P340+P310:** IF INHALED: Remove person to fresh air and keep comfortable for breathing. Immediately call a POISON CENTER/doctor; **P304+P340+P312:** IF INHALED: Remove person to fresh air and keep comfortable for breathing. Call a POISON CENTER/doctor if you feel unwell; **P305+P351+P338:** IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing; **P333+P313:** If skin irritation or rash occurs: Get medical advice/attention; **P362+P364:** Take off contaminated clothing and wash it before reuse.

Introduction

System Description

SepsiTest-UMD is CE-marked as an in vitro diagnostic medical device in accordance with Directive 98/79/EC. Where placed on the market on or after 26 May 2022, ***SepsiTest-UMD*** continues to be placed on the market in accordance with the applicable transitional provisions of Regulation (EU) 2017/746 (IVDR), meeting the conditions set out therein for devices compliant with Directive 98/79/EC.

SepsiTest-UMD is a kit for the detection of bacterial and/or fungal DNA in body fluids, swabs and tissues. In analogy to the recording of a metabolite in microbiological culture detection of pathogens, ***SepsiTest-UMD*** enables the detection of bacterial and fungal pathogens on a molecular level by the amplification of target sequences of rRNA genes. ***SepsiTest-UMD*** can detect pathogens up to days earlier than culture and in patients who are negative with culture (Kühn et al. 2011; Orszag et al. 2013).

Molzym's technology of depletion of host DNA, MolYsis, and isolation of bacterial and fungal 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 depletion of human DNA, enriched microbial 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-UMD is designed for broad-range detection of bacterial and fungal DNA based on conserved 16S and 18S rDNA target regions. Detectability depends on sequence compatibility, organism load, sample type, sample handling, DNA extraction efficiency and absence of PCR inhibition. Pathogens classified as safety level S3 or S4 are excluded. (please see 'Contraindication' on page 8), including both culturable and non-culturable strains. 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.

Test Principle

In its concept, ***SepsiTest-UMD*** is a means of molecular analysis of a broad range of clinical specimens for the presence of pathogens. The system combines new solutions for sample preparation and PCR analysis of clinical specimens, in particular EDTA-stabilised whole blood samples, other body fluids, swabs and tissues (Figure 2, page 17). 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 or melting curve analysis.

SepsiTest-UMD is based on two basic steps:
i) Pathogens are enriched from the sample after the depletion of the human DNA and microbial DNA is purified by removal of PCR inhibitors (tissue is additionally pre-treated to release pathogens from biofilms).
ii) The eluate is analysed by universal rDNA PCR for bacterial and fungal 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 body fluids, tissues and swab 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 (Figure 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*. Tissue samples are partially digested by a protease treatment before adding the chaotropic lysis buffer. 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 which enables the recovery of femtogram to picogram amounts of DNA in a 100 µl eluate.

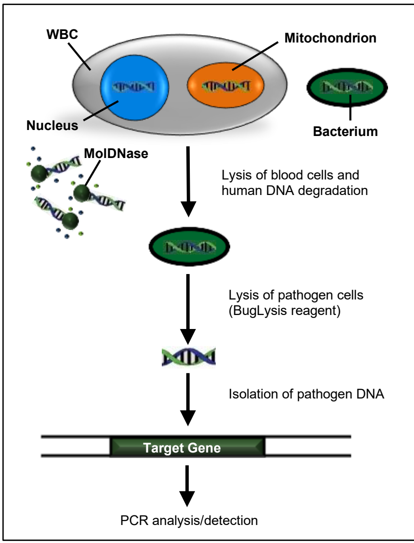


Figure 1 The principle of testing for bacterial and fungal DNA in samples by ***SepsiTest-UMD*** (blood as an example).

Part 2(a): PCR Analytics

DNA eluates (part 1, page 14) are used for broad-range 16S and 18S rDNA PCR analysis. Two assays are supplied, assay Bacteria (component *MA Bac*) and assay Yeasts (component *MA Yeasts*) 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. For testing the function of the extraction process and performance of the PCR assay regarding PCR inhibition by DNA extracts, a control DNA and control assay (component *MA Control*) is included in the kit.

Part 2(b): Pathogen Identification by Sequence Analysis

A protocol for sequencing of amplicons is supplied to identify detected pathogens. The

procedure includes a short protocol for amplicon purification and another for sequencing of amplicons. Primers for Sanger sequencing are supplied with this kit.

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 (Figure 2). 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 Bacteria (component *MA Bac*) employs primers, *SeqGN16* and *SeqGP16*.
SeqGN16 targets mainly Gram-negative bacteria and including few gram-positive bacteria. The excluding few gram-negative species will be detected by *SeqGP16*.
SeqGP16 targets mainly Gram-positive bacteria and including few gram-negative species. The excluding species will be detected by *SeqGN16*. The primers are not strictly discriminative. For the exceptions of the sequencing primers consult table on https://www.molzym.com/images/services/Exceptions_of_Sequencing_Primers.pdf.
By using both sequencing primers in separate reactions, mixed strains of the two groups can be resolved.
Sequencing of amplicons from the fungal assay (component *MA Yeasts)* is performed using *SeqYeast18*.
Bacterial taxa and fungal taxa (only *Candida* spp., *Cryptococcus* spp., and *Aspergillus* spp.) are identified with the help of the free online tool, SepsiTest-BLAST (https://www.sepsitest-blast.com). An overview of all species strains included SepsiTest-BLAST database is given in an Excel file which can be downloaded in the FAQ section of the SepsiTest-BLAST homepage (https://www.sepsitest-blast.com/en/faqs.php).

There are three potential outcomes of the sequencing analysis:
i) In case of readable sequences (Figure 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.
<u>Please note:</u> SepsiTest-BLAST is a quick tool for identification but does not contain all species. For comparison, we recommend to blast sequence results also with another tool e.g., NCBI BLAST https://www.ncbi.nlm.nih.gov/blast. The result with the best score is valid. For more information see section 2H) Analysis for Strain Identification on page 42.
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 (Figure 2, B, not readable); in such cases a service is available for identification of bacteria in mixed sequences (Pathogenomix, https://www.ripseq.com).
iii) If the amount of amplicon is too low for a sequencing reaction (Figure 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.

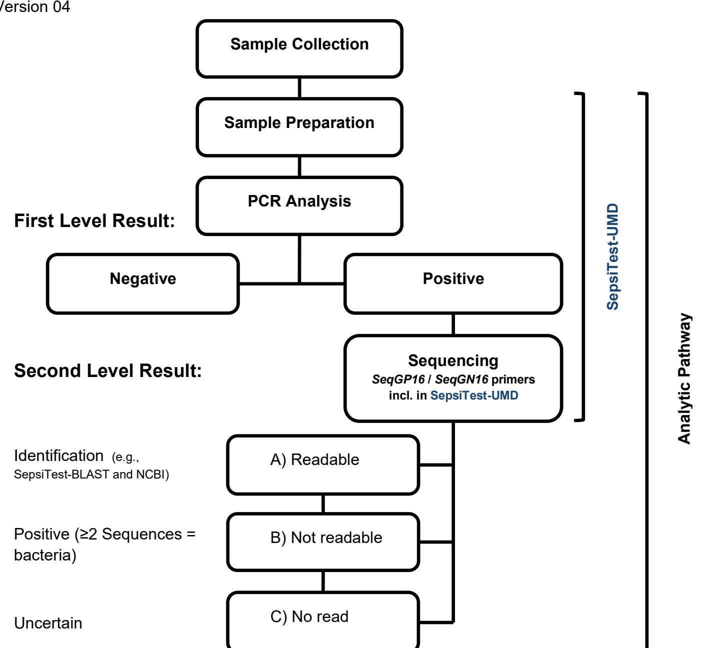


Figure 2: Detection of microorganisms in samples using ***SepsiTest-UMD*** and sequencing analysis. The analytic pathway includes the detection and identification of bacteria and fungi (only bacteria shown).

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.

Internal Extraction Control

The Internal Extraction Control testing must be added to each sample in the extraction process to validate the extraction of DNA.

The *Control DNA* (Internal Extraction Control Unit in Kit 2) is a DNA template to be used as a process control to monitor DNA extraction from samples and DNA quality as well as the absence of PCR inhibitors.

The kit supplies an assay (component *MA Control*, Kit 3) to which an aliquot of the eluate (including the *Control DNA*) is added. Generation of an amplicon indicates the correct function of the DNA extraction and purification process. Also, the absence of co-eluted PCR inhibitors is indicated.

Sample Controls

Positive Sample Control

Run Controls are not mandatory but recommended to run, e.g., once per reagent lot or in a determined time interval.
This control reflects the performance of the lysis and DNA extraction procedure from microorganisms and should be performed at least once per setup. There are two ways proposed to perform a positive sample control:
i) Negative samples (buffer *SU*, Kit 1) are spiked with 100 to 1000 cfu/ml of cultured Gram-negative (e.g., *E. coli*) or 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 BioBall® MultiShot 550 KBE (bioMérieux, Germany).

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 sample (buffer *SU*, Kit 1) is used and run through the extraction and detection protocols of this kit.

PCR Controls

PCR controls are included in the kit and are available in sufficient quantities for 2-3 samples per run.

Positive PCR Control

This test includes a definite number of target sequence copies to make sure that the assay is performing as specified. The *DNA Standard P1* (Kit 4B) comprises a mixture of DNAs extracted from *Bacillus subtilis* and *Saccharomyces cerevisiae*.
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 approx. 1.0 ng/µl (*MA Bac*) and approx. 0.1 ng/µl (*MA Yeasts*). Using this standard DNA indicates the functioning of the assays. The low concentrated DNA standard (P2) is diluted from P1 to approx. 2.0 pg/µl (*MA Bac*) and approx. 0.2 pg/µl (*MA Yeasts*) 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 998 µl of DNA dilution buffer in a 1.5 ml sterile polypropylene tube, add 2 µl DNA Standard P1 and vortex to mix. Always prepare P2 freshly for each series of PCRs. ***Do not re-use***, because diluted DNA solutions tend to be unstable.

Negative PCR 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.

Validation

Broad-range 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 one mismatch (excluding terminal sites). As a result, more than 345 species are detectable, among which more than 200 species have been sequence-identified by MolYsis technology in clinical evaluations so far (Table 5, page 21).

List of Strains Tested for Extraction

SepsiTest-UMD 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 bovis* (BCG), *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-UMD 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 deplete human DNA (‘Test Principle’, pages 14 to 17).

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 20 cfu/ml blood by the universal PCR assay. For other strains, see Table 4 (page 21) 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 Table 4, page 21.

Clinical Evaluation

A broad spectrum of Gram-negative, Gram-positive, and fungal organisms were identified in clinical samples using ***SepsiTest-UMD*** (Table 5, page 21).

Table 4: Analytical sensitivity of ***SepsiTest-UMD***.
Minimum titre resulting in positive results from 3 repeated extractions of fluid samples spiked with strains. Analysis: Real-Time PCR (5 µl 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

Table 5: Extract of microorganisms identified in clinical evaluations

Gram-negative bacteria	*Kluyvera* spp.	**Gram-positive bacteria**	*Microbacterium* spp.
Achromobacter spp.	*Lautropia mirabilis*	*Abiotrophia* spp.	*Micrococcus* spp.
Acidovorax spp.	*Legionella* spp.	*Actinomyces* spp.	*Mogibacterium timidum*
Acinetobacter spp.	*Leptothrix* spp.	*Aerococcus* spp.	*Mycobacterium* spp.
Aeromonas spp.	*Leptotrichia* spp.	*Aerosphaera* spp.	*Mycoplasma* spp.
Afipia spp.	*Massilia* spp.	*Alloiococcus otitis*	*Nocardia* spp.
Aggregatibacter aphrophilus	*Methylobacterium* spp.	*Amycolatopsis lurida*	*Nocardioides* spp.
Bacteroides spp.	*Moraxella* spp.	*Anaerococcus* spp.	*Paenibacillus* spp.
Bartonella spp.	*Morganella morganii*	*Anaerotruncus colihominis*	*Parvimonas micra*
Bilophila wadsworthia	*Morococcus cerebrosus*	*Arcanobacterium* spp.	*Peptococcus niger*
Bordetella spp.	*Necropsobacter rosorum*	*Atopobium* spp.	*Peptoniphilus* spp.
Borrelia garinii	*Neisseria* spp.	*Bacillus* spp.	*Peptostreptococcus* spp.
Bosea spp.	*Neoehrlichia mikurensis (Candidatus)*	*Bifidobacterium* spp.	*Rhodococcus* spp.
Brevundimonas spp.	*Pandoraea* spp.	*Blautia producta*	*Rothia* spp.
Brucella spp.	*Pantoea* spp.	*Brevibacterium* spp.	*Staphylococcus* spp.
Burkholderia spp.	*Paracoccus* spp.	*Carnobacterium* spp.	*Sarcina ventriculi*
Campylobacter spp.	*Pasteurella* spp.	*Clostridium* spp.	*Shuttleworthella satelles*
Capnocytophaga spp.	*Plesiomonas shigelloides*	*Coprococcus catus*	*Streptococcus* spp.
Chryseobacterium spp.	*Porphyromonas* spp.	*Corynebacterium* spp.	*Tissierella creatinini*
Citrobacter spp.	*Prevotella* spp.	*Dermabacter hominis*	*Tropheryma whipplei*
Cloacibacterium normanense	*Proteus* spp.	*Dermacoccus* spp.	*Tsukamurella* spp.
Comamonas spp.	*Providencia* spp.	*Dietzia* spp.	*Ureaplasma urealyticum*
Cronobacter spp.	*Pseudomonas* spp.	*Dolosigranulum pigrum*	*Vagococcus* spp.
Cupriavidus spp.	*Ralstonia* spp.	*Eggerthella* spp.	
Curvibacter spp.	*Rickettsia* spp.	*Enterococcus* spp.	**Fungi**
Delftia spp.	*Roseomonas* spp.	*Eremococcus coleocola*	*Aspergillus* spp.
Dialister spp.	*Salmonella* spp.	*Eubacterium* spp.	*Candida* spp.
Ehrlichia spp.	*Serratia* spp.	*Facklamia* spp.	*Cladosporium cladosporioides*
Elizabethkingia meningoseptica	*Shigella* spp.	*Finegoldia magna*	*Cryptococcus* spp.
Enhydrobacter aerosaccus	*Shewanella* spp.	Gardnerella vaginalis	*Davidiella tassiana*
Enterobacter spp.	*Stenotrophomonas* spp.	*Gemella* spp.	*Fusarium* spp.
Erwinia spp.	*Synergistes* spp.	*Gordonia* spp.	*Malassezia* spp.
Escherichia spp.	*Tannerella forsythia*	*Gordonibacter pamelaeae*	*Pseudallescheria* spp.
Flavobacterium spp.	*Undibacterium* spp.	*Granulicatella* spp.	*Rhodotorula hordea*
Francisella spp.	*Veillonella* spp.	*Janibacter* spp.	*Saccharomyces cerevisiae*
Fusobacterium spp.	*Vibrio* spp.	*Kocuria* spp.	*Schizophyllum* spp.
Haemophilus spp.	*Weeksella* spp.	*Lactobacillus* spp.	*Sporobolomyces* spp.
Hafnia alvei	*Wolbachia* spp	*Lactococcus* spp.	*Trichophyton* spp.
Helicobacter pylori	*Yersinia* spp.	*Leuconostoc* spp.	
Kingella spp.		*Leifsonia* spp.	**Protist**
Klebsiella spp.		*Listeria* spp.	*Plasmodium* spp.

Upon request, Molzym can provide the full list of “Microorganisms found in clinical and other specimens by sequencing” including Gram-positive and Gram-negative bacteria, fungi and protists down to species level and is constantly updated (except for pathogens of safety level S3 and S4).

Avoidance of DNA Contamination

Care should be taken to avoid DNA contamination from exogenous sources. This includes 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). A short summary of precautions is given below:

- **Guidelines**:
  The guidelines of the national health organisations, e.g., Robert-Koch-Institute (Germany), for sample collection, including sterilisation of the skin should be followed.

- **Decontamination**:
  Generally, for pre-analytical and analytical processing, use places decontaminated from DNA. We recommend performing 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.

- **Infectious material and cross-contamination**:
  Handle potentially infectious material with great care and work under a Class II biological safety cabinet to protect yourself from infection and avoid cross-contamination of samples and carry over contamination of extraction buffers and reagents.

  Wear a disposable lab coat, sterile protective gloves, sterile disposable sleeve covers and protective googles at any handling step, including handling of infectious material, sample pre-treatment and PCR analysis. Frequently change protective gloves during handling and change protective clothing 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 fluid.

  All DNA extraction buffers and reagents (DNA Isolation unit, Kits 1 and 2) are assembled in 4 sample extraction units to minimise the risk of contamination of buffers and reagents during handling. 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 11).

  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 (see page 18).

Part 1: Pre-Analytics

DNA Isolation

Use the following kits:

- ***Kit 1*** *(store at +18 to +25°C)*
  - *- Buffers (packages A, D and E)*
  - *- ST - Sample tubes (package B)*
  - *- SC - Spin columns (package C)*
  - *- CT - Collection tubes (package C)*
  - *- ET - Elution tubes (package C)*
- ***Kit 2*** *(store at -15 to -25°C)*
  - *- Enzymes and Reagents*
- ***Internal Extraction Control Unit,***
  in Kit 2 (store at -15 to -25°C)
  - *- Control DNA*

Part 1 - Pre-Analytics - DNA Isolation

Important notes before starting

Sample Collection

Because of the universal nature and the extreme sensitivity of detection of the assays, special care has to be taken for sample collection to avoid contamination by skin and environmental microorganisms. It is recommended to transfer the samples to the laboratory for immediate processing (pages 26 to 52). If this is not possible, store the samples in a refrigerator (+4 to +12°C). The stored sample must be analysed within two days after sample collection to avoid loss of microbial DNA.

! Use only fresh samples.

For blood collection, Molzym has evaluated K-EDTA and citrate S-Monovette® (Sarstedt, Germany) for the use with ***SepsiTest-UMD***.

Do not freeze samples to avoid potential loss of microbial DNA due to cell disruption because of freezing and thawing. If freezing of samples is desired, use a suitable cryoprotectant which stabilises fluid samples. Thaw samples to room temperature for extraction.

The procedure of pathogen DNA isolation can be interrupted at step 5, details on page 28.

General Information - Isolation of Pathogen DNA

! We strongly recommend a user training before using the kit for the first time.

Work in a place, which is ideally in a lab separated from places where mastermixes are handled and amplification is performed.

Wear sterile protective gloves, sterile disposable sleeve covers, a disposable lab coat and protective goggles when handling infectious material. Work in a Class II biological safety cabinet 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. Do not work under UV irradiation.

! For equipment, consumables and reagents to be supplied by the user see pages 10 to 11.

! To minimise cross-contamination, this unit is assembled in vials containing buffers or reagents for 4 sample extractions. 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 bottles/vials immediately after removal of solution. The vials of buffer *SU* are only intended for one sample each and must be discarded after use (incl. residual buffer).

! Take care that the enzymes and reagents of Kit 2 (*Enzyme K*, *MolDNase B, BugLysis, ß-mercaptoethanol* and *Proteinase K* solutions) 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).

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

! Before starting the protocol thaw the *Control DNA* (1 vial per 4 samples; Internal Extraction Control Unit, Kit 2) at room temperature (+18 to +25°C) (needed for step 8, page 28).

! Adjust the thermomixer to 37°C (needed for step 7, page 28).
Place vials of buffer *ES* (1 vial per 4 sample) into the thermomixer (needed for step 17, page 29). For tissue samples: Heat the thermomixer to 56°C for the pre-treatment (section 1B, iii. Tissue samples, page 27).

! Per sample, mark a *Spin column* (*SC*), two *Collection tubes* (*CT*, 2.0 ml) and one *Elution tube* (*ET*, 1.5 ml) of Kit 1C with a permanent marker for identification of samples.

! 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 12). Arrange all items according to your personal customs.

Procedure

1A) How to Start

- **Kit 1** (+18 to +25°C) and **Internal Extraction Control Unit** (Kit 2, -15 to -25°C).

  Kit 1 contains buffers and consumables (single packages A to E) for the extraction and isolation of DNA from patient samples.

  Internal Extraction Control Unit contains the *Control DNA* (in bags, -15 to -25°C).

  Open the packages and vials only in the Class II biological safety cabinet. Thaw the Control DNA at room temperature (+18 to +25°C; needed for step 8, page 28).

  Shortly centrifuge the buffer vials (Kit 1).

  Arrange all the vials for the needed sample numbers (Kit 1 and Internal Extraction Control Unit in Kit 2) in a separate rack according to the sequence of steps as below:

  SU (for fluid samples and swabs) or PKB – TSB (for tissue)

  CM – DB1 – RS – RL – Control DNA – RP – CS – AB – WB – WS – ES

  →

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

  The opened buffers of Kit 1A can be stored at room temperature (+18 to +25°C) in a dark, DNA-free place to the expiry date of the Kit.

  The opened buffer *SU* must be discarded. Each vial is intended for one sample.

- **Kit 2** contains the enzymes and reagents (in white boxes, -15 to -25°C).

  Take care that *Enzyme K*, *MolDNase B*, *BugLysis*, *2-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.

  Make sure that the enzymes are not frozen when pipetting. Before use, vortex the enzymes and shortly centrifuge the vials to clear the lid.

1B) Set-Up and Pre-Treatment of Samples

i) Fluid samples
(ascites, BAL, blood, CSF, plasma, pleural fluid, pus, synovial fluids)

Fluid specimens are sampled under conditions avoiding contamination (page 22) and transported to the laboratory.

For the isolation of blood samples use only K-EDTA- or citrate-stabilised whole blood (see ‘Sample Collection’ on page 25).

1. Pipette 1 ml fresh fluid sample from the sample container into a *Sample tube* (*ST tube*; Kit 1). In case of sample volumes less than 1 ml, pipette the fluid into the *ST tube* and fill up to 1 ml with buffer *SU* (Kit 1) (use the measure line of the tube).

Continue with step 1 of the DNA Isolation Protocol (section 1C; page 28).

ii) Swabs (mouth, nasopharynx, wounds, bones)

1. Pipette 1 ml of buffer *SU* (Kit 1) into a *Sample tube* (*ST tube;* Kit 1). If there is fluid in the swab vial, pipette 1 ml thereof into a *ST tube* instead of buffer *SU*. In case of less sample volume available, fill up to 1 ml by pipetting buffer *SU* to the sample in the *ST tube* (use the measure line of the tube).
2. Remove the swab from the swab vial and transfer to the *ST tube*. Wash the swab by swirling in the fluid and pressing to the wall of the *ST tube* several times. Thereafter discard the swab.

Continue with step 1 of the DNA Isolation Protocol (section 1C; page 28).

iii) Tissue samples (abscesses, biopsies, heart valves, prostheses)

Tissue specimens are sampled under conditions avoiding contamination (page 22) and transported to the laboratory.

1. Pipette 180 µl of buffer *PKB* (Kit 1) into a *Sample tube* (*ST tube*, Kit 1).
2. Transfer the specimen to a sterile support, e.g., a Petri dish, by using sterile forceps. For preparation of the tissue specimen, the area should measure at maximum approx. 0.5x 0.5 x 0.5 cm. Cut the specimen into small pieces by using a sterile scalpel or sterile preparation scissors.
3. Transfer the cut specimen to the *ST tube* filled with buffer *PKB*. The specimen should be covered completely by the buffer.
4. Add 20 µl of *Enzyme K* (Kit 2) to the specimen. Pipette the enzyme into the buffer.
5. Vortex the *ST tube* at full speed for 15 s and incubate in the thermomixer at 56°C and 1,000 rpm for 10 min.
6. After the incubation, adjust the thermomixer to 37°C.

   Comment: The tissue is partially digested and may decay. Potentially present bacteria and fungi are released from biofilm.
7. Fill up to 1 ml with the transport solution, if available, or with buffer *TSB* (use the measure line of the tube) and go to step 1 of the DNA Isolation Protocol (section 1C; page 28).

1C) DNA Isolation Protocol

1. **Pipette 250 µl buffer *CM* to the *ST tube* containing the sample (section 1B, page 27). Vortex at full speed for 15 s to mix properly. Let stand on the bench at room temperature (+18 to +25°C) for 5 min.**

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

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

2. **Briefly centrifuge to clear the lid. Pipette 250 µl buffer *DB1* to the *ST tube*. Thereafter pipette 10 µl *MolDNase B* to the lysate in the *ST tube*. Immediately vortex for 15 s properly and let stand for 15 min 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 10 min. Thereafter, carefully remove the supernatant by pipetting and discard.**

   Human cell debris and potentially present pathogen cells are sedimented.

4. **Pipette 1 ml buffer *RS* to the sediment and resuspend by pipetting.**
   Depending on the specimen, the sediment may contain residues of tissue and may be rigid due to debris. Resuspension may take some time.

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

   This washing step removes residual *MolDNase B* activity, chaotropic salts 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 80 µl buffer *RL* to the *ST tube*. Resuspend the sediment by vigorous vortexing. Briefly centrifuge to clear the lid.**

   Pipette 20 µl *BugLysis* directly into the extract in the *ST tube* and then add 1.4 µl *ß-mercaptoethanol*. Vortex the tube for 15 s and incubate in a thermomixer at 37°C and 1,000 rpm for 30 min. Store not 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.

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

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

7. **Note:** Preparation in this step, do not work in the sample tube itself.

   Pipette 10 µl of the *Control DNA* into a vial of buffer *RP* (4 samples) and vortex the tube for 15 s. Briefly centrifuge the vial to clear the lid. Continue to *ST* tube.

8. **Briefly centrifuge the *ST tube*. Add 150 µl buffer *RP* incl. the *Control DNA* to the *ST tube*. Then pipette 20 µl *Proteinase K* directly to the extract in the *ST tube*. Vortex the *ST tube* for 15 s and incubate at 56°C and 1,000 rpm for 10 min.**

   Store not used *Proteinase K* in the vial at -15 to -25°C and opened *RP* vials incl. *Control DNA* for up to 4 days (+18 to +25°C).

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

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

   Cells are lysed and protein is denatured.

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

11. **Briefly centrifuge the *ST tube* and transfer the lysate to a *Spin column* (*SC*; Kit 1, package C) pre-assembled in a 2.0 ml *Collection tube* (*CT*) by pipetting.** **<u>Tissue samples:</u> Do not transfer undigested material** (pulse centrifuge to sediment and pipette supernatant. You may want to use a smaller pipette tip to avoid transferring particles).
    Close the lid of the *Spin column* and centrifuge at ≥12,000x*g* for 30 s (or minimum time of the centrifuge, e.g. 60 s).

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

13. **Remove the closed *Spin column* from the centrifuge. Open the lid, remove the *Spin column and insert it into a new 2.0 ml Collection tube* (*CT; Kit 1, package C).* Discard the *Collection tube* with flow-through. Pipette 400 µl of buffer *WS* to the *Spin column*. Close the lid of the *Spin column* and centrifuge at ≥12,000x*g* for 3 min.**

    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 1.5 ml *Elution tube* (*ET*; Kit 1, package C). Discard the *Collection tube* containing the flow-through.**

15. **Place 100 µl buffer ES (vials in the thermomixer already preheated to 70°C), in the centre of the column, close lid and incubate for 1 min at room temperature (+18 to +25°C). Thereafter, centrifuge at ≥12,000xg for 1 min 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 30 to 42).**

    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 & Identification Unit:

- ***Kit 3*** *(store at -15 to -25°C)*
  - *PCR Reagents*
- ***Kit 4*** *(store at -15 to -25°C)*
  - *PCR Controls & Detection Reagents*
- ***Consumables PCR Detection & Identification (Kit 1)*** *(store at +18 to +25°C)**:***
  - *MT - Mastermix tubes, 1.5 ml*

Part 2 – Analytics

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.

Note: The term 'mastermix' refers to the mix of the components of Table 6, page 37. Assay Bacteria, Assay Yeasts and Assay Control refers to the assay of the analysis. The component names (*MA Bac*, *MA Yeasts* and *MA Control*) are also used as synonyms for the assays.

Description of the Assays

With this unit, PCR assays are supplied for the testing of the presence of bacterial and fungal DNA in clinical specimens. The PCR 'assay Bacteria' (*MA Bac*, Kit 3) and 'assay Yeasts' (*MA Yeasts*, Kit 3) are based on primers that bind to conserved regions of the 16S (V3/V4 region) and 18S (V8/V9 region) rRNA genes of bacteria and fungi, respectively.

The tests comprise a two-step procedure including the use of **i)** the assays (components *MA Bac* and *MA Yeasts*; Kit 3) for the PCR amplification of sequences using extracted DNA (pages 24 to 29) 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 44 to 52).

The components *MA Bac* and *MA Yeasts* are 2.5x concentrated solutions, the final volume of the reaction mixture being 25 µl. This PCR Detection unit contains all components necessary for PCR runs.

The PCR 'assay Control' (*MA Control*, Kit 3) is a control for the performance of the extraction process and to confirm the absence of co-eluted PCR inhibitors together with the sample DNA. The assay tests the presence of the *Control DNA* (Internal Extraction Control Unit), which is added in the extraction process and is included in the eluate of each sample. The correct function of the extraction process and the absence of PCR inhibitors potentially co-eluted with the target pathogen DNA are monitored by 'assay Control'.

Protocols for amplification are supplied for the following instruments:

- Thermal Cycler (protocol, page 38):
  - Mastercycler® Eppendorf
- Real-Time PCR instruments (addendum pages 45 to 47):
  - LightCycler® 96, 480 and Nano, Roche
  - DNA Engine Opticon®, CFX96™, BioRad
  - Mx3000P®, Mx3005P®, Stratagene
  - ABI 7500 Fast®, Life Technologies
  - Rotor-Gene®, Qiagen
  - peqStar 96Q, peqlab

If using other instruments, make sure that the Assays Bacteria, Yeasts and Control (*MA Bac*, *MA Yeasts* and *MA Control*) perform correctly with the cycler. For *MA Bac* and *MA Yeasts*, perform PCR reactions using PCR DNA Standard P1 and P2 which both should result in an amplification product. For *MA Control* a negative sample control (*SU* buffer, Kit 1) is extracted and tested in the assay. See PCR Detection, section 2A to 2E for the procedure (pages 34 to 41).

PCR Assays

Assay Bacteria (*MA Bac*) and Assay (*MA Yeasts*):
With each assay 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 19) must be included to control the performance of the assay. Follow the instructions for the performance of positive PCR controls (pages 34 to 38).

Assay Control (*MA Control*):
The Assay Control (*MA Control*) is an Internal Extraction Control test to check the performance of the extraction process and to confirm the absence of co-eluted PCR inhibitors together with the sample DNA. The assay must be performed with each sample testing (pages 34 to 38).

Validity of results:
Only if the negative PCR controls in all assays (*MA Bac*, *MA Yeasts* and *MA Control*) lack a PCR signal, the positive PCR controls (P1 and P2) and the Internal Extraction Control result in a band of specific size in the gel electrophoresis analysis, the results of the sample test can be considered valid results.

<u>Exceptional cases:</u>
If the sample lacks a PCR signal, the Internal Extraction Control is positive and the negative PCR controls of assays Bacteria and Yeasts (*MA Bac* or *MA Yeasts*) show a positive result, which may be the result of contamination by handling, pathogens are probably not present in the sample or below the detection limit. In this case the negative result of the sample is valid.

In case of a specific signal in the sample test and the Internal Extraction Control lacks a signal, the result of the sample is valid. In this case, all PCR controls must perform as expected.

Packaging, Storage and Handling

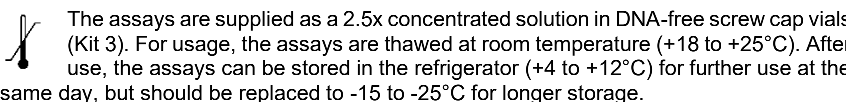
Store all vials in this unit (Kits 3 and 4) at -15 to -25°C upon receipt.

For more information about the storage and stability of the PCR reagents see Table 1 (page Storage and Stability6) and in section 2A) How to Start (page 34).

The purification and packaging of the assays supplied in this PCR detection unit are performed under standard precautions for the avoidance of air-borne and handling-based DNA contaminations.

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 microbial DNA-free pipette tips and PCR consumables recommended for running the assays (pages 10 to 11).

<u>*MA Bac*, *MA Yeasts* and *MA Control* (Kit 3)</u>



The assays are supplied as a 2.5x concentrated solution in DNA-free screw cap vials (Kit 3). For usage, the assays are thawed at room temperature (+18 to +25°C). After use, the assays 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/18S (Kit 3)

The enzyme must be kept at -15 to -25°C throughout handling (cooling rack). Replace *MolTaq 16S/18S* to the freezer (-15 to -25°C) after handling.

Make sure that the *MolTaq 16S/18S* is not frozen when pipetting. Before use, vortex the *MolTaq 16S/18S* and shortly centrifuge the vial to clear the lid.

Do not interrupt the cooling of MolTaq 16S/18S.

DS (including SYBR® Green1, Kit 3)

Once thawed, do not freeze again and store at +4 to +12°C for further use.

It is important to note that the DNA staining solution (*DS*, Kit 3) is sensitive to light and should be stored in the dark during handling and storage.

H_2O

For usage, the H_2O is thawed at room temperature (+18 to +25°C) and thereafter placed in cooling racks adjusted to -15 to -25°C. After use, the H_2O can be stored in the refrigerator (+4 to +12°C) for further use at the same day but must be replaced to -15 to -25°C for longer storage.

SM (including SYBR® Green1, Kit 4A)

Once thawed, do not freeze again and store at +4 to +12°C for further use.

It is important to note that the DNA size marker (*SM*, Kit 4A) is sensitive to light and should be stored in the dark during handling and storage.

LS (Kit 4A)

Store the *Gel loading solution* (*LS*, Kit 4A) after first handling at +4 to +12°C.

DNA dilution buffer and *DNA Standard P1* (Kit 4B)

Store *DNA dilution buffer* and *DNA Standard P1* (Kit 4B) at +4 to +12°C after thawing.

Quality Control and Specifications

Negative PCR controls using supplied DNA-free water instead of eluate are used for routine detection 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 ≤5 %, 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. *DNA Standard 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*.

2A) How to Start

! For equipment, consumables and reagents to be supplied by the user see pages 10 to 11.

Avoidance of DNA Contamination (for more details, see page 22)

! To avoid contamination, it is important that the setup of *MA Bac* and *MA Yeasts* is performed in a lab separated 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.

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** and **consumables** (*MT*, Mastermix tubes for Kit 3) in **Kit 1**.
For the preparation of mastermixes *MA Bac, MA Yeasts* and *MA Control*.

DNA handling

Work under a UV Class II biological safety cabinet where samples are prepared. Use components of **Kit 4B.** For the preparation of:

- Sample loading into the mastermixes
- Positive PCR controls P1 and P2

PCR Assays

Per assay (*MA Bac, MA Yeasts* and *MA Control*), the following PCR reactions must be run:

DNA-free

Assay Bacteria (*MA Bac*)

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

Assay Yeasts (*MA Yeasts*)

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

Assay Control (*MA Control*; *Internal Extraction Control*, IEC)

- 1 reaction per sample
- 1 reaction for negative control (NC IEC)

Before starting with the preparation of PCR-ready mastermixes (section 2B, page 37):

DNA-free

Kit 3:

- Thaw the following vials at room temperature (+18 to +25°C):
  - *H_2O*
  - *MA Bac* (2.5x conc.)
  - *MA Yeasts* (2.5x conc.)
  - *MA Control* (2.5x conc.)
  - *DS* (10x conc.;light sensitive)

  Vortex thawed PCR reagent vials (Kit 3) for a few seconds to mix and briefly centrifuge to clear the lid.

DNA

Kit 4B:

- Thaw the following vials at room temperature (+18 to +25°C):
  - *DNA Standard P1*
  - *DNA dilution buffer* (for P1)

- Preparation of Positive PCR Control P2 (P1 diluted 1:500)
  1. Vortex the *P1* and *DNA Dilution buffer* vials and pulse centrifuge.
  2. Pipette 998 µl *DNA dilution buffer* in a 1.5 ml polypropylene tube (not supplied).

  Tubes used must be DNA- and DNase-free (e.g., see page 10).

  3. Add 2 µl *P1* into the tube (with *DNA dilution buffer*) and vortex to mix.
  4. Briefly centrifuge to clear the lid.
  5. Label the dilution with ‘P2’ and the preparation date.

Storage of the PCR Reagents after Handling

For more information about the storage and stability of the PCR reagents see section Packaging, Storage and Handling (page 32) and Table 1 (page 7).

MA Bac, *MA Yeasts*, *MA Control* and *H_2O*
After use, keep in a refrigerator (+4 to +12°C) if reused at the same day.
Store at -15 to -25°C for longer periods.

MolTaq 16S/18S
Replace in a cooling rack (-15 to -25°C). Always keep and store at -15 to -25°C.

Do not interrupt the cooling of *MolTaq 16S/18S*.

*DS (*including SYBR® Green1)
After first use, store at +4 to +12°C. **Do not re-freeze**.

DS is sensitive to light and must be stored dark during handling and storage.

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

Positive PCR control *P2* (needed for step 8, page 37)
Diluted DNA solutions (P2) tend to be unstable. **Do not use** the *P2* for **longer than 24 hours** and store it at **+4°C to +12°C**.

2B) Setup of the Assays

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

DNA-free

Preparation of mastermixes

1. **Arrange the PCR tubes (strips or plates; not supplied) for all mastermixes (*MA Bac*, *MA Yeasts* and *MA Control*) in a PCR cooling rack (-15 to -25°C) and mark (PCR Assaying, page 35).**
2. **Briefly centrifuge *MolTaq 16S/18S* (Kit 3) and place in the cooling rack (-15 to -25°C).**
3. **Use a *MT* (*Mastermix tube* 1.5 ml for Kit 3; in Kit 1) for *MA Bac*, another *MT* tube for *MA Yeasts* and a third *MT* tube for *MA Control*.**
   Place the *MT* tubes in a cooling rack. Pipette the supplied components of Kit 3 into each *MT* tube as shown in Table 6. Vortex the tubes to mix and briefly centrifuge.
4. **Pipette 20 µl of the mastermix *MA Bac* into each PCR well dedicated for samples, P1, P2 and NC, respectively. Repeat the procedure with mastermix *MA Yeasts*. Pipette 20 µl of the mastermix *MA Control* into each PCR well dedicated for samples and NC.**
5. **Add 5 µl *H_2O* (DNA-free water; Kit 3) into the NC PCR wells. Close all PCR wells with caps.**

DNA handling

6. **Place the PCR strips/plate in another cooling rack (-15 to -25°C) designated for transport to the UV Class II biological safety cabinet.**

Sample loading (template loading)

7. **Pipette 5 µl of each sample eluate into the PCR wells containing *MA Bac*, *MA Yeasts* and *MA Control*, respectively. Close the PCR wells.**

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

8. **Vortex the *P1* and prepared dilution P2 (998 µl of *DNA dilution buffer* and 2 µl *DNA Standard P1*, see page 34f.) vials and pulse centrifuge. Pipette 5 µl of positive PCR control *P2* into a PCR well containing *MA Bac* and *MA Yeasts*, respectively. Repeat with *P1*. Close PCR wells with caps.**
9. **Continue with section 2C PCR Thermocycling page 38.**

Table 6: Preparation of mastermixes (Kit 3). Volumes in µl.

reactions	*MA Bac, MA Yeasts* or *MA Control*	*H_2O*	*DS*	*MolTaq 16S/18S*
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 or plates, -15 to -25°C) prepared as above to a place where PCR runs are performed. Program the Mastercycler (Eppendorf) as described (Table 7). After the PCR run go to section 2D for the detection of amplicons.

Table 7: PCR program 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 (strips or plates) to a place where DNA is handled. Use components of Kit 4A.

! For equipment, consumables and reagents to be supplied by the user (page 11).

! The DNA staining solution (*DS*, including SYBR® Green1), 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.

Before starting with the preparation of gel electrophoresis:

- Thaw the following vials at room temperature (+18 to +25°C):
  - *SM* (DNA size marker, 1 kb)
  - *LS* (Gel loading solution, 6 x conc.)

- Storage of components after handling:
  - *SM* (including SYBR® Green1, Kit 4A)

    Once thawed, do not freeze again and store at +4 to +12°C for further use.

    It is important to note that the DNA size marker (*SM*) is sensitive to light and must be stored dark during handling and storage.

  - *LS* (Kit 4A)

    Store the Gel loading solution (*LS*) after first handling at +4 to +12°C.

For more information about the storage and stability of the PCR reagents see page 6.

Protocol:

Prepare a gel (2 %) following the instructions of the manufacturer or prepare a 2 % (w/v) agarose 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).

1. Per PCR reaction, pipette 8 µl of the PCR product into a sterile 1.5 ml polypropylene tube (not supplied, DNA- and DNase-free) or in a well of a 96 well plate (not supplied, DNA- and DNase-free) and mix with 2 µl of the gel loading solution (*LS*). Mix by pipetting in and out for several times.

2. Pipette the mixture (10 µl) into an indentation of the gel. Repeat the procedure with the other PCR product, including samples, Internal Extraction Controls, positive PCR controls (P1 and P2), negative PCR controls and, at the end pipette 5 µl of supplied DNA size marker (*SM*; Kit 4A).
3. Close the electrophoresis chamber with the cover and run the gel at 10 V/cm interelectrode distance in the dark.

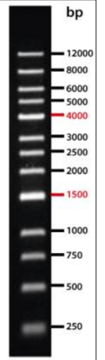


Figure 3: DNA size marker (*SM*, 1kb ladder) as a reference for gel electrophoretic detection of bacterial (*MA Bac*), fungal (*MA Yeasts*), and internal extraction control (*MA Control*) amplicons.

4. 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 45 min.
5. Remove the gel, place under a UV lamp or on a transilluminator (260 to 310 nm 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 Figure 9 and Figure 10, pages 40 to 41.
   Note: If all bands show a too weak fluorescence signal, the gel can be re-colored in e.g. an ethidium bromide staining bath, if necessary.
6. Make sure that bands appear within the samples in the Assay Control (*MA Control*; Internal Extraction Control). This is important in cases of negative samples. Bands in this assay indicate the absence of PCR inhibitors in the eluates and the correct performance of the extraction process.

2E) Guidance to the Interpretation of PCR Results

A typical image of the results of the analysis of samples is given in Figure 9 for the Assay Bacteria (*MA Bac*) and Figure 4 for the Assay Yeasts (*MA Yeasts*), including Assay Control (*MA Control*, Internal Extraction Control) in each figure (right side).

In this case, samples were collected from four patients and processed as described in the previous sections.
The controls are as expected, i.e., positive PCR controls P1 and P2 show bands at the correct position (approx. 450 bp for bacterial DNA in Figure 9 and appr. 310 bp for fungal DNA in Figure 5).
The negative PCR controls (NC) do not show a signal, so DNA contamination can be excluded.
The Internal Extraction Controls of the samples B1 through B3 (Figure 9) and samples Y1, Y3 and Y4 (Figure 6) show clearly visible bands (approx. 200 bp), demonstrating the function of the extraction process. The results from the Internal Extraction Controls, positive PCR controls and negative PCR controls indicate that the assay performed correctly.

Two of the samples show bands (samples B1 and B2) in the assay *MA Bac* at the expected gel position (Figure 9, left side) and thus are positive for bacterial DNA. Samples B3 (Figure 9) is negative with a positive signal in the assay *MA Control* (C3). This is a valid negative result for sample B3. Sample B4 was negative too, but no signal in assay *MA Control* (C4). This indicates inhibition in the sample and the result is invalid.

<u>Exceptional cases:</u>
In the case of specific bacterial or fungal bands in the samples tested (e.g., sample Y2, Figure 7) but lacking signal in the Internal Extraction Control (C2, Figure 8), the positive fungal result for the sample is valid.

All positive PCR samples must be identified by sequencing.

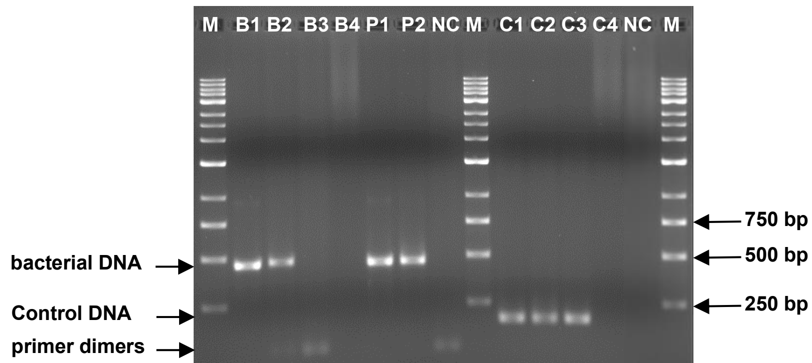


Figure 9: PCR analysis of eluates from four patient samples (B1 to B4) using Assay Bacteria (*MA Bac*). *P1, P2*: Positive PCR controls; NC: Negative PCR controls; C1 through C4: Internal Extraction Controls (*MA Control*) with respective eluates from patient samples B1 to B4 (banding at approx. 200 bp; C4: negative); M: DNA size marker (*SM*).
Signals at the position 'bacterial DNA' (450 bp) indicate that samples of patients B1 and B4 contain bacterial DNA (sequencing result: *Staphylococcus aureus*). The weak signals in sample B3 and NC at approximately 50 bp (arrow 'primer dimers') are the results of the unspecific binding in negative amplicons.

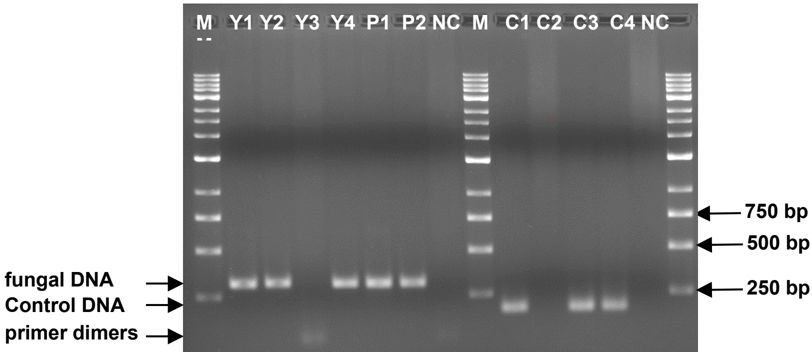


Figure 10: PCR analysis of eluates from four patient samples (Y1 to Y4) using Assay Yeasts (*MA Yeasts*, fungi). P1, P2: Positive PCR controls; NC: Negative PCR control; C1 through C4: Internal Extraction Controls (*MA Control*) with respective eluates from patient samples Y1 to Y4 (banding at approx. 200 bp; C2: negative); M: DNA size marker (SM). Signals at the position 'fungal DNA' (310 bp) indicate that samples of patients Y1, Y2 and Y4 contain fungal DNA (sequencing result: *Candida albicans*). The weak signals in sample Y3 at approximately 50 bp (arrow 'primer dimers') are the results of the unspecific binding in negative amplificants.

Identification of Pathogens by Sequencing of Amplicons

All positive PCR samples must be identified by sequencing.
Sequencing of amplicons together with BLAST online homology search is used for the identification of pathogens detected by ***SepsiTest-UMD***. Sequence analysis has been done with ***SepsiTest-UMD***. Other 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 and more than 340 sequences from cultured *Candida* spp, *Cryptococcus* spp. and *Aspergillus* spp. (https://www.sepsitest-blast.com). For further investigations see section 2H, page 42.

2F) Purification of Amplicons

For sequencing, amplicons need to be purified. Qiagen's QIAquick® PCR Purification Kit (cat. no. 28104) is recommended with ***SepsiTest-UMD***. For this purpose, use the aliquot remaining after analysis of the PCR product (approximately **17 µl**; 25 µl, if using Real-Time PCR; addendum pages 44 to 52) and follow the instructions of the manufacturer of the kit. Elute the purified amplicon from the column (QIAquick®) with **30 µl** sterile deionised water. Continue with the sequencing procedure (section 2G Sequencing, page 42).

2G) Sequencing

Apply the purified amplicon DNA to a sequencing reaction as advised by the manufacturer of the sequencing system. ***SepsiTest-UMD*** 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).

The BigDye® Terminator Direct Cycle Sequencing Kit cannot be used as the sequencing primers supplied with the ***SepsiTest-UMD*** kit do not contain the required M13 sequences.

For sequencing, use the sequencing primers (10 µM each) supplied with Kit 4. For sequencing of amplicons from 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. *SeqGN16* targets mainly Gram-negative bacteria, including few gram-positive bacteria. The excluding gram-negative species will be detected by *SeqGP16*. *SeqGP16* targets mainly Gram-positive bacteria, including few gram-negative species. The excluding species will be detected by *SeqGN16*. The exceptions of the sequencing primers are available at the following link:
https://www.molzym.com/images/services/Exceptions_of_Sequencing_Primers.pdf

As an example, the following protocol for QIAquick®-purified amplicons using the ABI Prism310® may give satisfying results. Use 2 µl of purified DNA for cycle sequencing. Add 4 µl Big-Dye® Reaction mix (containing polymerase und nucleotides), 0.5 µl sequencing primer *SeqGP16*, *SeqGN16* or *SeqYeast18* (10 pmol/µl) and PCR-grade water to fill up to a final volume of 20 µl. Incubate in a PCR machine under the following conditions: Initial denaturation at 95°C for 1 min; 26 cycles at 95°C for 30 s, 55°C for 30 s and 60°C for 4 min. To remove dye terminator molecules from sequencing samples, use your internal validated process. Combine 5 µl of the eluate containing the products of the sequencing reaction with 20 µl formamide (or TSR reagent containing formamide) and incubate at 95°C for 4 min. 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., Eurofins Genomics, Germany).

2H) Analysis for Strain Identification

Molzym has developed SepsiTest-BLAST, a free online service (https://www.sepsitest-blast.com), for the identification of bacteria and fungi based on small subunit rRNA genes. The identification relies on an algorithm for the comparison of input sequences with a reference sequence data library. SepsiTest-BLAST is characterized 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, *Candida* spp., *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.
An overview of all species covered by the SepsiTest-BLAST database is given in an Excel file which can be downloaded in the FAQ section of the SepsiTest-BLAST homepage (https://sepsitest-blast.com/en/faqs.php).
Please note: SepsiTest-BLAST is a quick tool for identification but does not contain all species. For comparison, we recommend to blast sequence results also with another tool e.g., NCBI BLAST https://www.ncbi.nlm.nih.gov/blast The result with the best score is valid.

Interpretation of the results:

Sequence identities ≥97 to <99 % should be interpreted on the genus level, ≥99 % on the species level (Wellinghausen et al. 2009). Furthermore, for an optimal

sequencing result, the coverage of the sequence should also be high (>99%) and the length of the aligned segment should be >350 bp for bacterial and >200 bp for fungal sequences. 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 (Figure 2, page 17). Overlying bacterial sequences can be resolved using a specialised tool, RipSeq® (Pathogenomix; https://www.ripseq.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-UMD*** does not provide a licence for the use of Real-Time PCR (see legal aspects, below). In the following, protocols are described for Roche LightCycler® 96, 480 and Nano Real-Time PCR machines, BioRad Opticon® DNA Engine and CFX96™ machines, ABI 7500 FAST, Stratagene Mx3000P® and Mx3005P® machines, Qiagen Rotor-Gene® and Peqlab peqStar 96Q. Other instruments may be validated for their use with this kit by the user. At the end, (pages 47 to 52) 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 30 to 41). 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. In particular, the patents for real-time PCR and the use of intercalating fluorescent dyes and probes, including their specific applications.

General Requirements

Please take notice of the general requirements for the performance of PCR (part 2, pages 30 to 38).

! Calibrate your Real-Time PCR machine using the Assays Bacteria, Yeasts and Control (*MA Bac*, *MA Yeasts* and *MA Control*; Kit 3). For *MA Bac* and *MA Yeasts*, perform Real-Time PCR runs with supplied *Standard DNA P1* and *P2*. For *MA Control* a negative sample control (*SU* buffer, Kit 1) is extracted and tested in the assay.

The PCR run conditions are as according to the protocol described on pages 34 to 38. The specific thermocycling conditions are described on pages 45 to 47. For the preparation of mastermixes, follow the instructions (part 2, sections 2A to 2C, pages 34 to 38). Both positive PCR controls, P1 and P2 (*MA Bac* and *MA Yeasts*), and the sample eluate (*MA Control*) must show a target-specific peak (see page 47).

! For equipment, consumables and reagents to be supplied by the user see pages 10 to 11. In addition, the following items are needed to perform Real-Time PCR:
- 1x Real-Time PCR machine (above). Other instruments must be validated for their use.
- PCR tubes (strips or plates), e.g., OCR strips (8x 0.2 ml) for various systems; e.g., Biozym Flat Optical 8-Cap Strip (order no. 712100)

! To avoid contamination, it is important that the setup of *MA Bac*, *MA Yeasts* and *MA Control* is performed in a lab separated from DNA extraction and PCR amplifications.

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 bacterial or fungal signals. See examples on pages 47 to 52. All positive Real-Time PCR sample results have to be identified by sequencing. For sequencing of amplicons see part 2, section 2F) Purification of Amplicons to 2H) Analysis for Strain Identification, pages 41 to 43.

I) 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	-	0.2	5	Continuous
Cooling	1	None	40	00:00:10	-	-	-

II) 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	-	-	-

III) 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	

IV) BioRad DNA Engine Opticon® and CFX96™

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		

V) ABI 7500 Fast®

Switch off the ROX reference.

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 %	

VI) 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	00:00:15	
		72	00:00:30	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

VII) Stratagene Mx3000P® and Mx3005P®

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) Peqlab peqStar 96Q

Method	Cycles	Target Temperature [°C]	Incubation time [hh:mm:ss]	Ramp rate [°C/s]	Step	Step Holding Sec.
Hold Stage	1	95	00:01:00	4		
PCR Stage	40	95	00:00:05	4		
		55	00:00:10	4		
		72 (Sampling)	00:00:25	4		
Melting Stage	1	95	00:00:01	4	0.1	00:01
		70	00:00:01	4		
		95 (Sampling)	00:00:01	4		
Infinite Stage	1	8	∞	4		

Guidance to the Interpretation of Real-Time PCR Results

This kit supplies assays for the amplification of the 16S and 18S rRNA genes of eubacteria and fungi, 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 prone to false positive results due 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 (see examples pages 49 to 52).

Validity of results:

Only if the negative PCR controls (*MA Bac*, *MA Yeasts* and *MA Control*) lack a PCR signal, the positive PCR controls (P1 and P2) and the Internal Extraction Control result in a specific peak in the melting curve analysis, the results of the sample test can be considered valid results.

Exceptional cases:

If the sample lacks a PCR signal, the Internal Extraction Control is positive but the negative PCR controls *MA Bac* or *MA Yeasts* show a positive result, which may be the result of contamination by handling, pathogens are probably not present in the sample or below the detection limit. In this case the negative result of the sample is valid.

In case of a specific signal in the sample test and the Internal Extraction Control lacks a signal, the result of the sample is valid. Note that in this case all PCR controls must perform as expected.

Result Interpretation of Internal Extraction Control (*MA Control*):

The Assay *MA Control* (Internal Extraction Control; Kit 3) is a test to check the performance of the DNA extraction process and to confirm the absence of co-eluted PCR inhibitors together with the sample DNA. *MA Control* must be performed with each sample testing (pages 34 to 38).

For the interpretation of the Assay results use only the melting curve analysis and ignore

the Ct values (amplification curve).
The temperature of specific and potentially unspecific peaks depends on the used Real-Time PCR instruments. In the following sections examples are presented using the Roche LightCycler®96. Here, the specific peak of the Internal Extraction Control is located at approximately 90°C (Figure 6, page 49, blue melting curves). It is important to calibrate other Real-Time PCR instruments for the specific temperature of the Internal Extraction Control peak (see part 'General Requirements' page 44).

The specific peak can vary in height (part 2 of Figure 66, page 48; pink melting curves). In some cases, eluates of the samples can show one or two unspecific peaks (part 1 of Figure 66, page 48, red and green melting curves). In all cases, a distinct peak must show up in the specific temperature range of the Internal Extraction Control (e.g., part 1 of Figure 6, page 48, blue, red and green melting curves) for valid results.

Absence of a peak in the specific temperature range indicates a negative result of the Internal Extraction Control assay. In this case, the results are invalid and the extraction must be repeated.

Exceptional case: In case of a specific signal in the sample test and absence of a peak in the Internal Extraction Control, the result of the sample is valid. In this case all PCR controls must perform as expected.

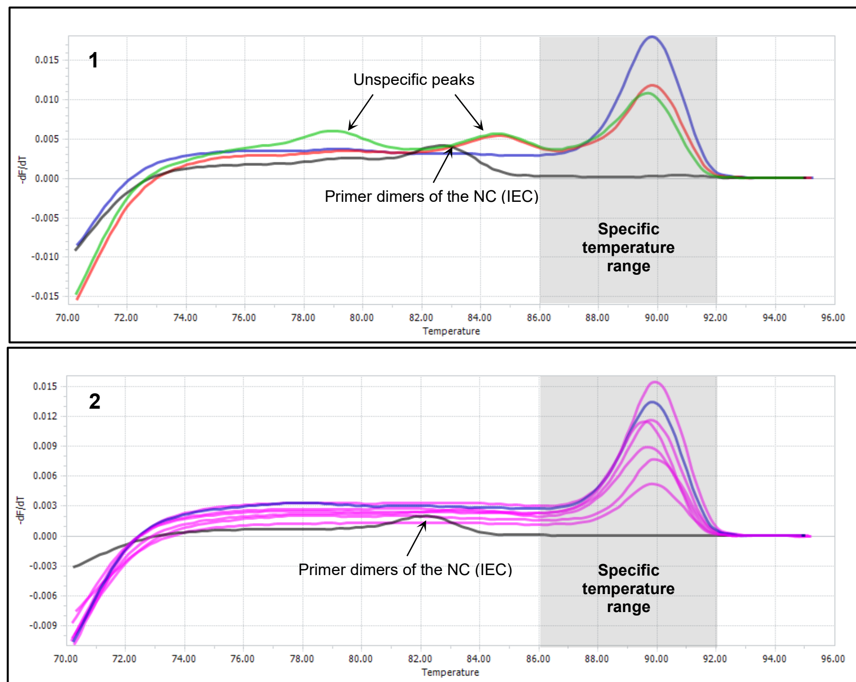
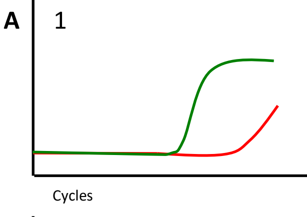


Figure 6: Melting curve analysis (Roche LightCycler® 96) of a negative reference Internal Extraction Control (NC (IEC), black curve) and eluates of samples showing different peaks (colored curves) in assay *MA Control*. The blue sample curve shows a specific peak (90°C) of the Internal Extraction Control. Part 1: The red sample curve shows the specific peak and an unspecific peak (85°C). The green sample curve shows three peaks (specific peak

at 90°C and two unspecific peaks at 85°C and 79°C). Part 2: The pink curves show the variety in height of the specific peak. All results are valid.

Schematic Examples for the Interpretation of Real-Time PCR Results:
Examples (A to H, pages 50 to 52) of Real-Time PCRs are shown in a schematic modus of amplification curves (1, upper image) and melting curves (2, 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 color code in the table corresponds to the curves in the images. For *MA Control* (IEC and NC IEC) only the melting curve analysis is shown.
In the examples below, it is given that the positive PCR controls (P1 and P2) indicate full functioning of the assay (not illustrated).
Legend to pages 50 to 52:

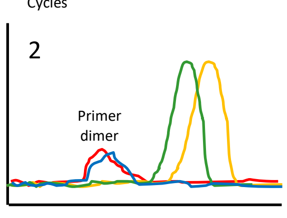
Sample: Mastermix Assay Bacteria (*MA Bac*) or Mastermix Assay Yeasts (*MA Yeasts*) – green curve; **NC (sample)**: Negative PCR controls – red curve (*MA Bac / MA Yeasts*); **IEC**: Mastermix Assay Control (*MA Control*, Internal Extraction Control) – yellow curve; **NC (IEC)**: Negative reference Internal Extraction Control – blue curve (*MA Control*); **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.

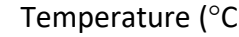


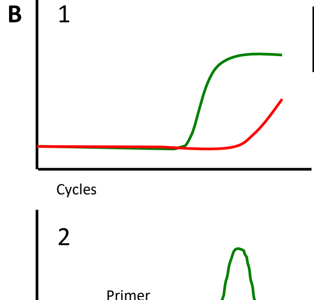
Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
+	-	+	-	+

True positive result

The Internal Extraction Control (IEC) appears at the expected value. The reference Internal Extraction Control (NC IEC) is negative as expected. The sample is positive in the melting curve analysis and the negative PCR controls (NC´s) do not show a signal (besides primer dimers).
The positive sample result is valid.





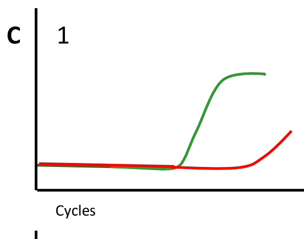


Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
+	-	-	-	+

True positive result

The sample is positive in the melting curve analysis. The Internal Extraction Control (IEC) lacks a signal in the melting curve, because the *Control DNA* was not added in the extraction process or the Internal Extraction Control (IEC) PCR setup was incorrect. The negative controls (NC´s) do not show a signal (besides primer dimers).
In this case the positive sample result is valid.

2
Primer
dimer
Temperature (°C)



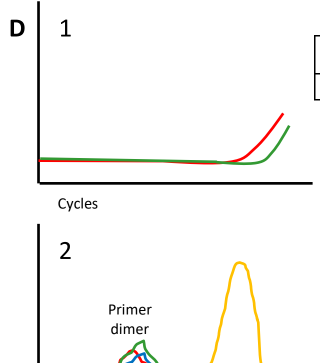
Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
+	-	+	+	+

True positive result

The sample is positive in the melting curve analysis, and the negative control (NC sample) does not show a signal (besides primer dimers). The Internal Extraction Control (IEC) is correct, but the reference Internal Extraction Control (NC IEC) shows a signal, indicating contamination in the *MA Control*. The positive result of the sample is correct.
In this case the positive sample result is valid.

2
Primer
dimer
Temperature (°C)

D

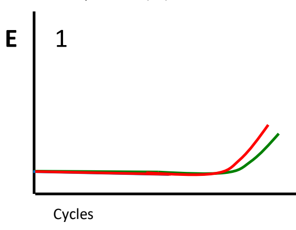


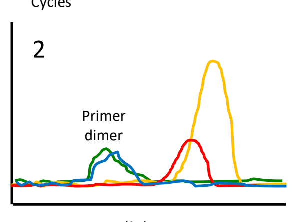
Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
-	-	+	-	-

True negative result

The Internal Extraction Control (IEC) is at the expected value. The sample, negative control (NC) and reference Internal Extraction Control (NC IEC) lack a peak in the melting curve analysis (only primer dimers). Hence, pathogens are not present or below the detection limit.
The negative sample result is valid.

E



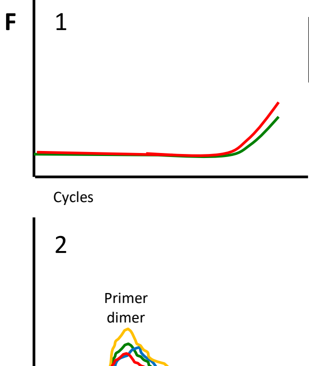


Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
-	+	+	-	-

True negative result

The Internal Extraction Control (IEC) is regular, and the sample lacks a signal. Despite a signal in the PCR negative control (NC sample), which may be the result of contamination by handling, pathogens are probably not present in the sample or below the detection limit.
The negative sample result is valid.

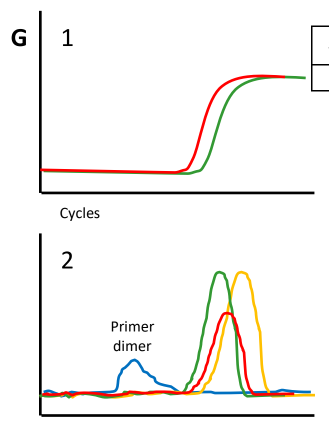
F



Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
-	-	-	-	?

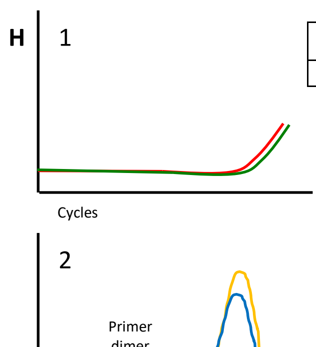
Invalid result

The PCR negative control (NC) and reference Internal Extraction Control (NC IEC) do not show a signal (besides primer dimers). The sample and the Internal Extraction Control (IEC) lack a signal (besides primer dimers) the latter indicating a failure in DNA extraction or PCR inhibition.
In such a case, the results are invalid and extraction must be repeated.



Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
+	+	+	-	?

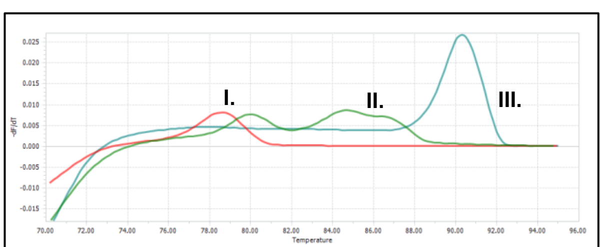
False positive result
The Internal Extraction Control (IEC) is regular, and the reference Internal Extraction Control (NC IEC) does not show a signal (besides primer dimers). However, the PCR negative control (NC sample) shows a signal, indicating contamination. It is not clear whether the signal of the sample comes from contamination or from a pathogen in the sample.
The PCR has to be repeated with more care to avoid contamination.



Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
-	-	+	+	?

Invalid result
The sample and the negative control (NC sample) do not show a signal (besides primer dimers). The Internal Extraction Control (IEC) is regular, but the reference Internal Extraction Control (NC IEC) shows a signal, indicating contamination in the *MA Control*. It is not clear whether the signal of the Internal Extraction Control (IEC) comes from contamination in the PCR mix or the extraction performed correctly.
The assay Internal Extraction Control (*MA Control*) must be repeated with more care to avoid contamination.

An example for an image of melting curve analysis (*MA Bac*):



Melting curve analysis (Roche LightCycler® 96) 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.).

Supplementary Information

Troubleshooting

This guide may help solve problems that may arise. For further support:

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Observation	Possible cause	Comments/suggestions
Weakly visible bands on agarose gel	• *DNA staining solution* (*DS*) not sufficient	The *DNA staining solution (DS)* added in the mastermix could be too weak to stain the bands sufficiently. In this case, re-stain the DNA in the gel to increase visibility of the bands. For example, use ethidium bromide according to manufacturer's instructions.
Strong human DNA background in gel electrophoresis 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 (Figure 9 and Figure 10, pages 40 to 41). 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.
	• *MolDNase B* volume too low	Ensure that *MolDNase B* vial is briefly centrifuged before use. Make sure that the enzymes are not frozen when used.
	• *MolDNase B* incorrect usage conditions	Keep the *MolDNase B* vial chilled, because warming may reduce enzyme activity and hence human DNA background may remain high.
	• Solutions not mixed properly	It is important that solutions are thoroughly mixed after addition of buffers. Follow instructions for vortexing.
No pathogen DNA detectable in spiking tests with *SU* buffer	• Insufficient lysis	Make sure that *BugLysis* and *ß-mercaptoethanol* have been added. Ensure that the *Proteinase K* treatment has been done. Ensure that all enzyme vials are briefly centrifuged before use. Make sure that the enzymes are not frozen when used.
	• PCR inhibition	Run the Internal Extraction Control assay (*MA Control*) for testing for proper DNA extraction. Inhibitors like ethanol are co-eluted. The result of *MA Control* was negative. Repeat the extraction.
	• Insufficient homogenisation	If the pellet at steps 4 and 7 (page 28) is not homogenised, pathogen cells may be included in the debris and not reached by lytic enzymes. Follow the instructions.
	• Insufficient DNA isolation	If white precipitate is visible in buffer CS, please vortex buffer CS and briefly centrifuge to clear the lid before use.
	• Pathogen load too low (below limit of detection)	Check the load 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 11, page 29). Accordingly, make sure that the column has been washed with buffer WB (step 13, page 29).
	• Loss of nucleic acids during the storage of the eluate	Store the eluted DNA at +4 to +12°C if analysed within 48h or freeze at -15 to -25°C until further use. Avoid frequent freeze-thaw cycles, because this may result in a decay of the eluted DNA (in particular at low DNA concentrations).
	• Wrong elution conditions	Make sure to elute with supplied heated buffer ES (70°C; step 16, page 29). This increases the DNA yield significantly.
No signal in PCR	• *MolTaq 16S/18S* not added • DS not added • *MA Bac*, *MA Yeasts* or *MA Control* not added • H_2O not added	Make sure that all reagents of the PCR-ready mastermixes have been added. Ensure that all reagents (except *MolTaq 16S/18S*) were completely thawed at room temperature and vortexed before use.Make sure that the *MolTaq 16S/18S* is not frozen when pipetting.
False positive PCR 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 22). Use DNA-free pipette tips and other plastics only as recommended (page 11).
False negative PCR result (no signal in Assay *MA Control*, Internal Extraction Control)	• PCR inhibitors co-eluted	Check whether *Proteinase K* treatment has been performed during DNA preparation and vials are briefly centrifuged before use. Make sure that all washing steps of the procedure have been followed. Optionally, after buffer *WS* washing (step 14, page 29), centrifuge for another 1min to avoid ethanol carryover to the eluate.
	• Mistakes in the extraction	Make sure that the *Control DNA* was added to buffer *RP* before using the buffer (steps 8 to 9, page 28).

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