Micro-Dx™

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

Body fluids

(e.g. *ascites, BAL, blood, CSF, joint aspirates, plasma, synovial fluid, urine*)

Swabs

(e.g. mouth, nasopharynx, wounds, bones)

Tissues

(e.g. *abscesses*, *biopsies, heart valves, prostheses*

CE IVD

– For *in-vitro* diagnostic use –

molzym
reveal DNA

Version 05

Date of first release: 11/2017
Last update: 01/2023

Contents page

Kit Information

Kit Contents – *Micro-Dx™*

Automated DNA Isolation	**24 rxn (U-200-024)**	**48 rxn (U-200-048)**
Kit 1 - Buffers & Consumables (store at +18 to +25°C)		
A) Sample Dilution & Tissue Pre-treatment Buffers, in rack		
SU	1x 25 ml	2x 25 ml
TSB	1x 25 ml	2x 25 ml
PKB	1x 7.5 ml	2x 7.5 ml
B) Cartridges & Consumables		
ST - Sample tubes, 2.0 ml, flip cap tubes for swabs & enzymatic pre-treatment only, in DNA-free bags	1x 50	1x 50
Plus-SV – Plus-Sample vials; screw cap vials for instrument, 2.0 ml, in DNA-free bags	2x 12	4x 12
Extraction columns, in DNA-free bags	2x 12	4x 12

Continued on next page

Extraction cartridges, in trays	2x 12	4x 12
Buffer cartridges, pre-filled, in trays	2x 12	4x 12
ET - Elution tubes, 1.5 ml, in DNA-free bags	2x 12	4x 12
Kit 2 – Enzymes (store at -15 to -25°C), in white boxes		
2A) *MolDNase C*, solution, red cap, in bags	2x [12x 0.05 ml]	4x [12x 0.05 ml]
2B) *Proteinase K*, solution, blue cap, in bags	2x [12x 0.04 ml]	4x [12x 0.04 ml]
2C) *BugLysis plus*, solution, yellow cap, in bags	2x [12x 0.02 ml]	4x [12x 0.02 ml]
2D) *Enzyme K,* solution, in bags	2x [3x 0.08 ml]	4x [3x 0.08 ml]
PCR Detection and Identification	**24 rxn**	**48 rxn**
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]
MA Yeasts, Mastermix assay yeasts, 2.5x conc.	2x 0.30 ml	2x [2x 0.30 ml]
MA Control, Mastermix assay control, 2.5x conc.	1x 0.36 ml	2x [1x 0.36 ml]
MolTaq 16S/18S	3x 0.05 ml	2x [3x 0.05 ml]
H_2O - DNA-free PCR-grade water	3x 0.75 ml	2x [3x 0.75 ml]
DS - DNA staining solution, 10x conc.	2x 0.30 ml	2x [2x 0.30 ml]
Kit 4 - PCR Controls & Detection Reagents (store at -15 to -25°C)		
A) Detection Reagents (in white box)		
LS - Gel loading solution, 6x conc.	1x 0.4 ml	1x 0.4 ml
SM - DNA size marker	1x 0.24 ml	1x 0.24 ml
SeqGP16 - Sequencing primer (bacteria)	1x 0.1 ml	2x 0.1 ml
SeqGN16 - Sequencing primer (bacteria)	1x 0.1 ml	2x 0.1 ml
SeqYeast18 - Sequencing primer (fungi)	1x 0.1 ml	2x 0.1 ml
B) Positive PCR Control (in transparent bag)		
DNA Standard P1, for PCR positive control runs	1x 0.3 ml	1x 0.3 ml
DNA dilution buffer (for P1)	1x 25 ml	2x 25 ml
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
Manuals (in Kit 1)		
Manual	1x	1x
Short manual sheets	5x	5x

Symbols

Symbols used in labelling and in section 'Hazard and Precautionary Statements' (pages 10 to 11).

For *in-vitro* diagnostic use


This product fulfils the requirements of the European Directive 98/79 EC for *in-vitro* diagnostic medical devices.


Content of the package


Manufactured by


Temperature limitation (store at)

Consult instructions for use

Batch code

Catalogue number


Use by


Flammable


Corrosive


Toxicity


Irritant


Health hazard


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. Guarantee for full performance of ***Micro-Dx™*** as specified in this manual is only valid if storage conditions are followed (Tab. 1).

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

Kit 1 (Buffers & Consumables): Buffers, cartridges and consumables must be stored dry, dark and at room temperature (+18 to +25°C).
Kit 2 (Enzymes): Take care that the vials of the DNA Isolation unit have to be stored at -15 to -25°C upon delivery.
Kit 3 (PCR Reagents) and kit 4A (Detection Reagents): Take care that the vials of the PCR Detection unit (Kits 3 and 4A) have to be stored at -15 to -25°C upon delivery. 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. ***Do not freeze again*** (*DS*, *SM* and *LS*) and store 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.
Kit 4B (Positive PCR Control): Please take care that the components of the Positive PCR Control Detection unit (Kit 4B) have to be stored at -15 to -25°C upon delivery and must be stored at +4 to +12°C after the first handling.

Tab. 1: Storage of the *Micro-Dx™* components (*exp. date: expiration date of the kit).

Components	Storage Temperature	Working Temperature	Storage & Stabilitiy after use Temperature	Days (dark)*
Kit 1 - Buffers & Consumables:				
SU	+18 to + 25°C	+18 to + 25°C	+18 to + 25°C	exp. date
TSB	+18 to + 25°C	+18 to + 25°C	+18 to + 25°C	exp. date
PKB	+18 to + 25°C	+18 to + 25°C	+18 to + 25°C	exp. date
Buffer cartridges	+18 to + 25°C	+18 to + 25°C	+18 to + 25°C	exp. date
Extraction columns	+18 to + 25°C	+18 to + 25°C	+18 to + 25°C	exp. date
Extraction cartridges	+18 to + 25°C	+18 to + 25°C	+18 to + 25°C	exp. date
Tubes (*ST* tubes, *Plus-SV* vials, *ET* tubes)	+18 to + 25°C	+18 to + 25°C	+18 to + 25°C	exp. date
Kit 2 - Enzymes & Reagents:				
Enzyme K, MolDNase C, BugLysis plus, Proteinase K	-15 to -25°C	-15 to -25°C	-15 to -25°C	exp. date
Kit 3 - PCR Reagents:				
MA Bac, MA Yeasts, MA Control	-15 to -25°C	+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	-15 to -25°C	+18 to + 25°C	+4 to + 12°C -15 to -25°C	1 (thereafter freeze) exp. date
DS (DNA Staining Solution)	-15 to -25°C	+18 to + 25°C	+4 to + 12°C	exp. date
Kit 4A - Detection Reagents:				
LS	-15 to -25°C	+18 to + 25°C	+4 to + 12°C	exp. date
SM	-15 to -25°C	+18 to + 25°C	+4 to + 12°C	exp. date
SeqGP16, SeqGN16, SeqYeast18	-15 to -25°C	+18 to + 25°C	-15 to -25°C	exp. date
Kit 4B - Positive PCR Control:				
P1 DNA Standard	-15 to -25°C	+18 to + 25°C	+4 to + 12°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	+18 to + 25°C	+4 to + 12°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

Micro-Dx™ 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. **Micro-Dx™** is intended for body fluids, swabs and for tissues (validated specimens see Tab. 6, page 19). The kit is for laboratory use (professional users).

Micro-Dx™ is a kit for sample analysis of patients with suspected bacterial or fungal infection.

Contraindication

Micro-Dx™ 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 **Micro-Dx™** are not used as the sole basis for diagnosis, treatment, or other patient management decisions.
Micro-Dx™ is not indicated for pathogens with safety level S3 and S4. An exemplary selection is listed in Tab. 2.

Tab. 2: Contraindication of **Micro-Dx™** for pathogens with safety level S3 and S4 (exemplary selection).

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

Product Use Limitations

Usage of **Micro-Dx™** 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. **Micro-Dx™** is not intended for frozen and thawed specimen materials, nor for highly viscous specimen materials. Not for other specimens than mentioned above. Cells must be intact for reliable results. This requires specimens not to be stored in solutions, which induce cell lysis. Transport media including Agar, gel, charcoal medium and Amies hold a risk of inhibiting the amplification or clogging the extraction columns and should be avoided. Test procedures must always be run including the control assay provided with this kit.

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.
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.

Necessary equipment for the instrument:

- **SelectNA™*plus*** DNA extraction instrument (Molzym order no. D-400-001).
- ***Pipette tips* SelectNA™*plus***, DNA-free (Molzym order no. D-925-024 / D-925-048 / D-925-096).
  Use only Molzym´s DNA-free *Pipette tips* for the SelectNA™*plus*.
- ***Waste bags* SelectNA™*plus***, (Molzym order no. D-928-500).

Sample preparation:

- 1x UV Class II biological safety cabinet
- 1x vortexer, e.g., VWR, Darmstadt, Germany
- 1x thermomixer (2.0 ml tubes), e.g., Eppendorf comfort, Eppendorf
- 1x low speed mini-centrifuge (e.g., MiniFuge, VWR, Darmstadt, Germany) or a bench top microcentrifuge (e.g., miniSpin, Eppendorf, Germany)
- Sample positive control (run control):
  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)
- Precision pipette up to 10 µl, up to 20 µl, up to 200 µl and up to 1000 µl, e.g., Eppendorf, Germany
- Sterile forceps for *Extraction columns* loading
- Sample racks

Only tissue protocol

- Sterile forceps
- Sterile support, e.g., Petri dish
- Sterile scalpel or sterile preparation scissors

PCR amplification:

- 1x UV PCR workstation, e.g., GuardOne® Werkbank, Starlab, Germany
- 1x low speed mini-centrifuge (≤2000x*g*) e.g., MiniFuge, 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 45 to 50)
  Optional: Real-Time PCR instruments are validated (pages 55 to 57)
- 1x 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

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 (300V, 500mA), 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:

- Purification of amplicons, Qiagen, QIAquick® PCR Purification Kit (28104)
- DNA sequencing apparatus,
  e.g., DNA Analyzers ABI 310, ABI 3130, ABI 3730XL, ABI 38730
- 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:

- DNA-free pipette tips (with aerosol filter), e.g., Biosphere® plus, Sarstedt, Germany
  - 10 µl type Eppendorf (70.1114.210)
  - 100 µl type Eppendorf (70.760.212)
  - 300 µl type Eppendorf (70.3040.255)
  - 1000 µl type Eppendorf (70.3050.255)
- 1.5 ml micro tubes, e.g., Biosphere®, 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)
- Surface decontamination, e.g., Meliseptol® New Formula (rapid disinfectant, ethanol containing), B. Braun, Germany (19758) or an ethanol containing disinfectant for cleaning of the SelectNA™*plus* instrument
- Cleaning of the waste chute: Mildly alkaline cleaning powder for special washing machines with sodium hydroxide, e.g., LABWASH® Premium Classic, VWR Chemicals (84548.410)
- Cleaning of the pipetting tubes: 1 % (active Cl_2) sodium hypochlorite solution, prepared from e.g., sodium hypochlorite 14 % Cl_2 in aqueous solution, VWR Chemicals (27900.296)
- Autoclaved deionized water (121°C, 1bar, 30 min) for the pipetting tubes.
- Disposables
  - Lab coat, e.g., VWR, Germany
  - Sterile gloves, e.g., Kimberly-Clark, Germany
  - Sterile sleeves, e.g., Cardinal Health, Ireland
  - Bouffant covers, e.g., VWR, Germany
  - Hygiene mask, e.g., VWR, Germany
  - Overshoes, e.g., hygi, Germany
- Waste container for plastics and liquid waste, autoclavable

Safety Information

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

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

The lysis buffer (W0) and binding buffer (W6) are pre-filled in the *Buffer cartridges*. These buffers contain guanidine hydrochloride and guanidinium thiocyanate, respectively, which can from 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. Aerosols created during the extraction process in the SelectNA™*plus* instrument could contain pathogens. Therefore, the opening of the door can be a risk for the user. In the end of the extraction process a 5 min UV step is implemented for more safety of the user. The UV step reduces this risk. Nevertheless, suitable protective clothing is essential when working in the instrument.

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, 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. Irradiate the SelectNA™*plus* using the instrument's programme for UV decontamination after each extraction run (see section 1D, page 40). Dispose of potentially infectious material and the waste including cartridges and vials following national directives 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 *PKB*

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

Warning

Hazard and precautionary statements*(page 11):
H302-H315-H319-H332; P280-P301+P312-P304+P340+P312-P305+P351+P338

Proteinase K, Enzyme K

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

Danger

Hazard and precautionary statements*(page 11):
H317-H334;
P280-P302+P352-P333+P313-P363

BugLysis plus

Contains 2-mercaptoethanol (<10 %): **Acute toxicity (skin), eye damage, skin sensitization, reproductive toxicity and hazardous to aquatic environment (chronic).**

Danger

Hazard and precautionary statements*(page 11):
H310-H317-H318-H361d-H411;
P273-P280-P301+P310-P302+P352+P310-P305+P351+P338

Lysis buffer, prefilled in *Buffer cartridges* (W0)

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

Warning

Hazard and precautionary statements*(page 11):
H302-H315-H319;
P301+P312-P302+P352-P305+P351+P338

Control buffer, prefilled in *Buffer cartridges* (W5)

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

Warning

Hazard and precautionary statements*(page 11):
H302-H315-H319-H332;
P280-P301+312-P304+P340+P312-P305+P351+P338

Binding buffer, prefilled in *Buffer cartridges* (W6)
Contains 2-propanol (<40 %); guanidinium thiocyanate (>10 %): **Flammable liquids, acute toxicity (oral, skin), skin corrosive and irritating (eyes), specific target organ toxicity (single exposure) and hazardous to aquatic environment (chronic)**.

Danger

Hazard and precautionary statements*:
H225-H302-H312-H314-H319-H336-H412-EUH032;
P210-P233-P280-P303+P361+P353-P305+P351+P338-P310-P362+P364

Washing buffer, prefilled in Buffer cartridges (W7)
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 center 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; **H411:** 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; **P301+P310:** IF SWALLOWED: Immediately call a POISON CENTER/doctor; **P363:** Wash contaminated clothing before reuse; **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+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

Micro-Dx™ is a molecular tool for the fully automated procedure of pathogen enrichment and isolation of microbial DNA from body fluids, swabs and tissue specimens and identification of bacteria and fungi by 16S and 18S sequencing PCR.
Micro-Dx™ is used in combination with the **SelectNA™*plus*** instrument which provide a new, completely automated solution for the depletion of host DNA, enrichment of microorganisms from clinical samples and the isolation of pure microbial DNA.
In analogy to the recording of a metabolite in microbiological culture detection of pathogens, ***Micro-Dx™*** enables the detection of bacterial and fungal pathogens on a molecular level by the amplification of target sequences of rRNA genes. ***Micro-Dx™*** can identify pathogens up to days earlier than culture and in patients who are negative with culture (Kühn et al. 2011; Meyer et al. 2014, Orszag et al. 2013). The fully automated extraction of pathogen DNA reduces the hands-on time significantly as compared to the manual extraction.

Molzym has developed a technology, *MolYsis™,* which comprises a procedure for the degradation of human DNA before extraction of microbial DNA and isolation of pathogen target DNA from human samples. This technology is combined with universal rDNA PCR assays providing a high quality, straight forward kit for molecular pathogen detection.

By the enzymatic degradation of human DNA, pure pathogen DNA is provided to the assays, minimising false results from unspecific primer binding. Furthermore, the high quality of all reagents of the kit guarantees tolerable reagent-borne false positive signalling (≤5 %) under the precautions of the avoidance of DNA contamination.

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

Test Principle

In its concept, ***Micro-Dx™*** 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 (Fig. 2, page 15). The procedure includes a fully automated DNA extraction of samples followed by 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.

Micro-Dx™ is based on two basic steps:
i) Pathogens are enriched from the sample after the degradation of the human DNA and then microbial DNA is purified by removal of PCR inhibitors (tissue needs a short additional pre-treatment to release pathogens from biofilms).
ii) The eluate is analysed by universal rDNA PCR for pathogen DNA. Sequence analysis of amplicons together with BLAST search leads to the identification of pathogens.

Part 1: Pre-Analytics (the automated SelectNA™*plus* procedure)
Micro-Dx™ kit supplies all reagents and consumables (excluding pipette tips, waste bags) for the automated extraction of microbial DNA from clinical samples. The ***Micro-Dx™*** kit is used with the **SelectNA™*plus*** instrument for the isolation of bacterial and fungal DNA

from 1 ml EDTA or citrate blood, other body fluid samples, platelet concentrates and swabs. For tissue biopsies a short manual protocol precedes the automated extraction of microbial DNA. After deposition of the sample into the instrument, the following protocol proceeds fully automated.

The pre-analytic procedure is based on the following four steps A to D (Fig. 1):

A In a first step, the sample (1 ml) is treated with a chaotropic buffer which lyses the host but not microbial cells and degrades the released host DNA (and any potentially present floating DNA from dead microorganisms) by a DNase. The lysate is liquefied and then passed through a filter column by vacuum filtration which retains microorganism on the filter potentially present in the sample.

B In a series of following steps the immobilized microbes are washed and lysed by enzymatic treatment.

C In the following steps the microbial DNA is bound on the filter and washed.

D Finally, the microbial DNA is eluted with 200 µl elution buffer. At the end, a microbial DNA preparation is available which is depleted of human DNA and can be used for molecular analysis.

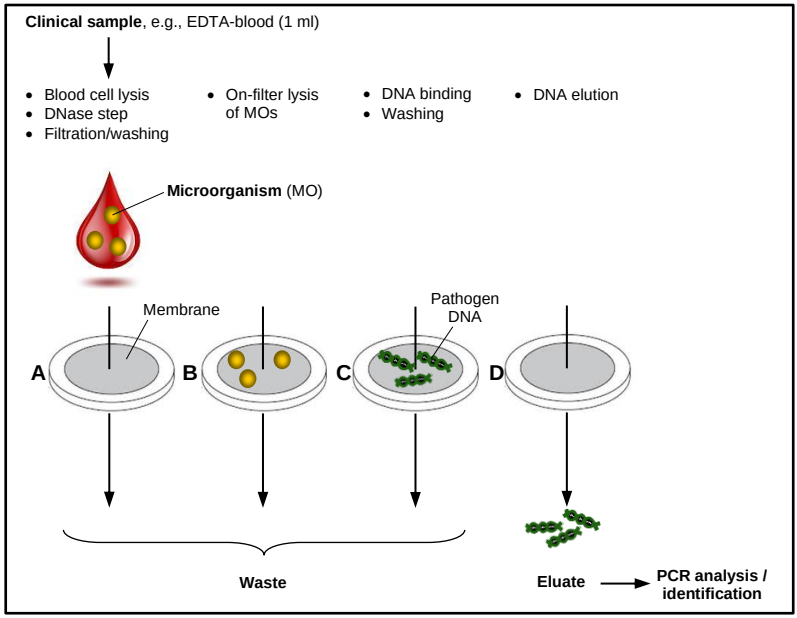


Fig. 1: Scheme of the fully automated **Micro-Dx™** procedure.

Part 2(a): PCR Analytics
DNA eluates (part 1, page 38) are used for broad-range 16S and 18S rDNA PCR analysis. Two assays are supplied, assay *MA Bac* (bacteria) and assay *MA Yeasts* (fungi) enabling the sensitive detection of pathogens. Protocols for the detection of amplicons by agarose gel electrophoresis and Real-Time PCR are supplied. For testing the performance of the extraction process and the PCR assay regarding PCR inhibition by DNA extracts a control assay (*MA Control*) is included in the kit.

Part 2(b): Pathogen Identification by Sequence Analysis
A protocol for sequencing of amplicons is supplied in order to identify detected pathogens. The procedure includes a short protocol for amplicon purification and another for sequencing of amplicons. Primers for sequencing are supplied with this kit.

Many routine applications demand high sensitivity and specificity analysis of bacterial and fungal pathogens in clinical specimens. The analysis of specimens using ***Micro-Dx***™ is a two-step generation of data (Fig. 2; page 15). After sample extraction, at the first step negative or positive results are obtained by PCR or Real-Time PCR analysis, indicating the absence or presence of bacterial and fungal target sequences in the sample. If positive, the second step of the analysis encompasses sequence analysis of amplicons. Sequencing is a way of confirming a positive PCR result and of gaining information on the identity of an organism detected. Therefore, sequence analysis should always follow PCR detection of amplicons. Sequencing of amplicons from assay *MA Bac* (bacteria) employs primers, *SeqGN16* and *SeqGP16*.
SeqGN16 targets mainly Gram-negative bacteria and including few gram-positive bacteria. The excluding few gram-negative species will detect 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. The exceptions of the sequencing primers see in 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, *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 (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 (http://www.sepsitest-blast.com/en/faqs.html).

There are three potential outcomes of the sequencing analysis:

i) In case of readable sequences (Fig. 2, A, readable; page 15), 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.
Please note: For comparison, we recommend to blast sequence results also with another tool e.g., NCBI BLAST http://www.ncbi.nlm.nih.gov/blast. The result with the best score is valid.

ii) If SepsiTest™-BLAST analysis indicates poor quality, overlapping sequences may be the reason as a result of more than one target sequence present in the reaction (Fig. 2, B, not readable; page 15); in such cases a service is available for identification of bacteria in mixed infections (Pathogenomix, www.ripseq.com).

iii) If the amount of amplicon is too low for a sequencing reaction (Fig. 2, C, no read; page 15), the result is considered negative (below the limit of sequencing detection). In this case, another analysis of a new sample should be performed, if possible.

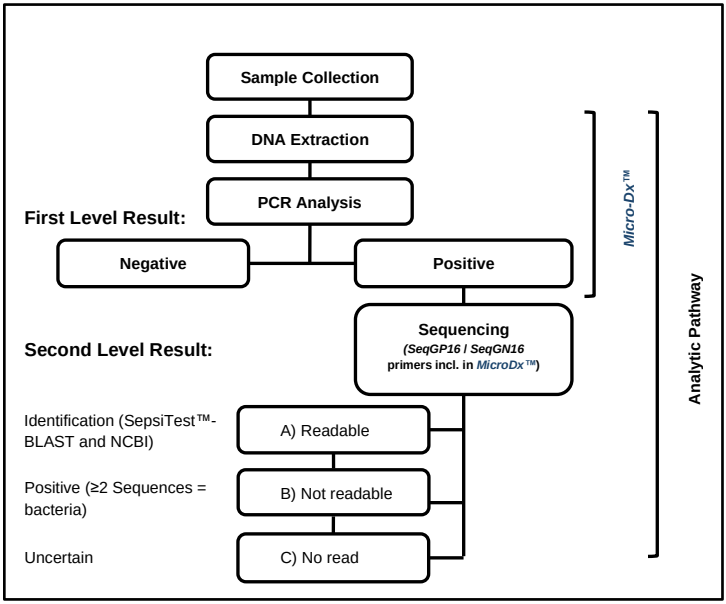


Fig. 2: Detection of microorganisms in samples using ***Micro-Dx™*** and sequencing analysis. The analytic pathway includes the detection and identification of bacteria and fungi (only bacteria shown).

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 performed with each sample in order to validate the extraction of DNA. The *Buffer cartridge* contains the Internal Extraction Control DNA which is passed through the extraction process ending up in the eluate.

The Internal Extraction Control is a DNA template 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 (*MA Control*, Kit 3) to which an aliquot of the eluate 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 Control)

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 run 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 organisms (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 successfully 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

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 (*MA Bac*) and approx. 0.1 ng (*MA Yeasts*) target DNA/µl. Using this standard DNA indicates the functioning of the assays. The low concentrated DNA standard (P2) is diluted from P1 to 2.0 pg/µl (*MA Bac*) and 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 (not supplied), add 2 µl DNA Standard P1 and vortex to mix. Always prepare P2 freshly for each series of PCRs. ***Do not re-use***, because dilute DNA solutions tend to be unstable.

Negative PCR Control (Reagent Control)

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

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 1 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 (Tab. 3).

Tab. 3: Extract of microorganisms identified in clinical evaluations.

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

Analytical Specificity

Micro-Dx™ includes two analytical 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 solved by the pre-analytical treatment of samples to deplete up to 99 % of human DNA (‘Test Principle’, pages 12 to 15).

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, *Staphylococcus aureus* can be detected reliably at 50 cfu/ml by ***Micro-Dx™***. Spiking experiments using negative samples and serial dilutions of cultured strains of clinical isolates showed the detection limits indicated in Tab. 4.

Tab. 4: Analytical sensitivity of ***Micro-Dx™***.
Titre resulting in positive results from 4 to 12 repeated extractions of buffer samples spiked with strains. Analysis: Real-Time PCR (5 µl eluate/assay; Assays: *MA Bac* and *MA Yeasts*) with melting curve analysis. Bold: Limit of detection at 100 % positives lowest dilution.

Strain	**cfu/ml detected (positive result)**													
Gram-negative bacteria														
Escherichia coli			**1,000**	100%	500	92%	250	78%	100	50%				
Klebsiella pneumoniae	5,000	100%	1,000	100%	500	100%	**250**	100%	100	75%				
Pseudomonas aeruginosa					**500**	100%	250	75%						
Gram-positive bacteria														
Enterococcus faecalis			200	100%	**100**	100%	50	25%	25	75%	5	0%		
Staphylococcus aureus	500	100%	**250**	100%	100	83%	50	67%						
Streptococcus agalactiae					100	100%			10	100%	5	100%	**1**	100%
Fungi														
Candida albicans									5	100%	**2,5**	100%	1	67%
Candida glabrata					20	100%	10	100%	**5**	100%			1	75%
Candida krusei	**100**	100%	50	75%	20	75%								
Candida parapsilosis					20	100%	10	100%	**5**	100%	2,5	67%		
Candida tropicalis					20	100%	10	100%	5	100%			**1**	100%

Clinical Evaluation

Micro-Dx™ was evaluated at several routine diagnostic laboratories in 5 European countries using a total of 409 samples (Tab. 5). Reference methods included manual CE IVD marked *SepsiTest™-UMD*, in-house used amplification assays and/or culture results. Median Sensitivity, Specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV) and Concordance of positive and negative results were 88 % (range: 79 to 100 %), 91 % (61 to 100 %), 89 % (19 to 100 %), 88 % (64 to 100 %) and 87 % (68 to 97 %). In 7 of the 9 evaluations, ***Micro-Dx™*** found microorganisms in samples at rates up to 32 % that were negative with the reference methods (Tab. 5). Among these, pathogens like *Candida parapsilosis* (heart valve), *Coxiella burnetii* (aortic biopsy), *Gemella taiwanensis* (aortic valve), *Haemophilus parainfluenzae* (aortic biopsy), *Neisseria meningitidis* (blood culture), *Propionibacterium avidum* (splenic biopsy), *Rhodococcus* spp. (prothesis), *Staphylococcus aureus* (various samples) and pyogenic and non-pyogenic streptococci (various tissues and aspirates) were identified by ***Micro-Dx™*** (not shown).

Tab. 5: Results of ***Micro-Dx™*** evaluations using samples from routine diagnosis (see Tab. 6). Legend of the data: Median Sensitivity, Specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV) and Concordance of positive and negative results.

Evaluation	Samples (n)	Reference method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Concordance (%)	Micro-Dx™ pos, reference neg (%)
Germany	66	SepsiTest™-UMD	82	92	88	88	88	6
Germany	33	SepsiTest™-UMD	93	100	100	95	97	0
Germany	110	SepsiTest™-UMD	82	92	89	86	87	5
Germany	64	In-house PCR, culture	88	75	54	95	78	19
Germany	21	SepsiTest™-UMD	80	91	89	83	86	5
Denmark	41	In-house PCR, culture	100	66	19	100	68	32
Switzerland	19	SepsiTest™-UMD, culture	94	100	100	64	95	0
France	37	culture	79	61	55	82	68	24
UK	18	In-house PCR, culture	100	88	91	100	94	6

Tab. 6: ***Micro-Dx™*** is validated with the following specimens.

Fluid samples	Pericardial effusion	Biopsy sphenoid sinus
Ascites aspirates	Urine	Bone marrow
BAL	**Swabs**	Heart valve biopsies
Blood	Bones	Hematoma
Blood cultures	Mouth	Lung tissues
CSF aspirates	Nasopharynx	Mucus
Joint aspirates	Wounds	Pacemakers
Liver abscess puncture fluid	**Tissue samples**	Paraffin blocks
Plasma	Abscesses	Pericard
Platelet concentrates	Aorta	Prostheses
Pleural fluid	Artificial tissues	(e.g. heart valve, …)

Do not use other specimens than validated in Tab. 6.

In particular, the following sample materials are inappropriate for the ***Micro-Dx™*** protocol:

- Gelatinous samples (e.g., sputum)
- Cell cultures
- Blood cultures with activated carbon
- Swabs on agar gel media
- Samples with transport media including Agar, gel, charcoal medium and Amies

These materials may clog the Pipette tips and Extraction columns which will cause the Pressure Monitoring System (pages 27 to 28) to reject the sample to prevent an overflow and contamination of the instrument and other samples.

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, use places decontaminated from DNA for handling. 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 transport supplies (e.g., pipettes, microcentrifuges, vortexer) and disposable material as specified by the handlings below from one working place to another. The place near the instrument should be equipped with a freezer (-15 to -25°C) for storage of the enzymes of the kit. The Class II biological safety cabinet should stand in the same room, optimally beside or near the instrument.
  Always UV-decontaminate the instrument after usage. Follow the instructions given below (section 1D, page 40).

- **Infectious material and cross-contamination**:
  Handle potentially infectious material with great care and work under a Class II biological safety cabinet in order to protect yourself from infection and to avoid cross-contamination of samples and carry over contamination of buffers *SU*, *PKB* and *TSB*. Wear a disposable lab coat, sterile protective gloves, sterile disposable sleeve covers, protective goggles and a disposable mask at any handling step, particularly when handling infectious material. Take care to open the enzyme vials (Kit 2, vials 2A through 2D) in the instrument.

- **SelectNA™*plus* instrument**:
  The instrument is a contained environment for contamination-free extraction and isolation of microbial DNA. The instrument has a UV source decontaminating the interior surfaces and air. Further, the instrument contains a Pressure Monitoring System (pages 27 to 28) which shall detect residual liquid in case of clogged columns and prevent an overflowing and subsequent contamination of the instrument and other samples. Loading of the instrument with the samples and supplied consumables, including columns, cartridges and enzyme vials, and pipette tips (to be ordered separately; order no. D-925-096) should be performed with care to avoid handling-borne contamination. Wear a disposable lab coat, protective gloves, arm sleeves and a disposable mask. Details for loading the instrument are given in the following chapters.

- **Pipette tips**:
  Use only Molzym´s DNA-free *Pipette tips* for the **SelectNA™*plus*** instrument (order no. D-925-024 / D-925-048 / D-925-096).

Part 1: Automated Microbial DNA Isolation

Use the following kits & components:

- ***Kit 1*** *(store at +18 to +25°C)*
  - *ST – Samples tubes* (flip cap tubes) for tissues & swabs pre-treatment only
  - *Plus-SV – Plus-Samples vials* (screw cap vials) for instrument
  - *Extraction columns*
  - *Extraction cartridges*
  - *Buffer cartridges*
  - *ET – Elution tubes*
- ***Necessary components (not supplied with this kit)***
  - *Pipette tips* (Molzym order no. D-925-0xy)
  - *Waste bags* (Molzym order no. D-928-500)

- ***Kit 2*** *(store at -15 to -25°C)*
  - *Enzymes*

Part 1 – Automated Microbial DNA Isolation

Important notes before starting

! Performance of the instrument as specified is guaranteed only if run conditions are followed (**+18 to +25°C**).

! A Class II biological safety cabinet should be near the instrument.

! For equipment, consumables and reagents to be supplied by the user see pages 7 to 8.

! Take care that *MolDNase C, BugLysis plus, Proteinase K* and *Enzyme K* vials (Kit 2) are stored in a freezer (-15 to -25°C) until usage.

Caution: 2-mercaptoethanol is a toxic compound included in *BugLysis plus* vial (yellow capped). Take care not to inhale and otherwise come into contact with when removing the cap.

! Use only fresh samples.
For blood collection, Molzym has evaluated K-EDTA and citrate S-Monovette® (Sarstedt, Germany) for the use with ***Micro-Dx™***. After collection, the sample should be transported to the laboratory and processed immediately. If this is not possible, the sample should be placed in a refrigerator (+4 to +12°C), where it can be stored for 2 days at maximum. ***Do not freeze samples*** to avoid potential loss of microbial DNA due to cell disruption as a result of freezing and thawing. If freezing of samples is desired, use Molzym's *UMD-Tubes* (order no. Z-801-020) which stabilise fluid samples. Thaw samples to room temperature for extraction.

! **Caution**: The following sample materials are inappropriate for the SelectNA™*plus*, because they may clog the *Pipette tips* and the *Extraction columns*:

- Gelatinous samples (e.g., sputum)
- Cell cultures
- Blood cultures with activated carbon
- Swabs on agar gel media
- Samples with transport media including Agar, gel, charcoal medium and Amies

Do not use sample materials of this kind in the instrument to prevent clogging of the *Extraction column* and subsequently rejection of the sample position.

! **Caution**: Wear sterile protective gloves, sterile disposable sleeve covers, a disposable lab coat, protective goggles and a disposable mask 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 of the sample preparation (section 1B, page 24).

Sample Collection

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 22 to 42). If this is not possible, store the samples in a refrigerator (+4 to +12°C). The stored samples should be analysed within 2 days after sample collection to avoid loss of microbial DNA. For longer storage of fluid samples, Molzym offers *UMD-Tubes* (order no. Z-801-020) which contain a cryoprotectant.

Procedure

Caution: Wear sterile protective gloves, sterile disposable sleeve covers, a disposable lab coat, protective goggles and a disposable mask 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.

1A) How to start

- **Kit 1** contains buffers (in *Buffer cartridges* and bottles) and consumables for the extraction and isolation of DNA from patient samples.

  Open the buffer bottles only in the Class II biological safety cabinet. The Class II biological safety cabinet should stand near the instrument!

  The following used buffer bottles and the unused consumables should be stored at room temperature (+18 to +25°C) in a dark, DNA-free place:

  - Buffers: *SU*, *TSB* and *PKB*
  - *ST tubes* for tissues & swabs pre-treatment only (flip cap tubes, 2.0 ml).
  - *Plus-SV* vials for instrument (screw cap vials).
  - *Extraction columns*
  - *Extraction cartridges*
  - *ET tubes* (*Elution tubes*, flip cap tubes, 1.5 ml)

! Preparation for automated DNA isolation (procedure, section 1C, pages 26 to 38):

For each extraction, use the following tubes:

- For each sample place an *ET* tube (*Elution tube*) in a rack, close the lid and mark the tube with the sample ID.
- For each sample place a *Plus-SV* vial (screw cap) in a rack. Mark the vial with the sample ID. **Caution:** Do not mark the *Plus-SV* vials on the lid, but rather the tubes on the white printed label.
- Only for swabs and enzymatic pre-treatment (tissue and diverse kind of fluid samples, see section 1B, page 24) use the *ST* tube (flip cap). Mark the tubes with the sample ID.

  Caution: Do not use the *ST* tubes in the instrument.

Continue with section 1B, page 24.

- **Kit 2** contains the enzymes.

  Take care that *MolDNase C*, *BugLysis plus, Proteinase K* and *Enzyme K* solutions are stored in a freezer (-15 to -25°C) until usage.

  For each extraction, use a vial each of *MolDNase C*, *BugLysis plus* and *Proteinase K*. **Briefly centrifuge the enzyme vials**, place them in a rack and store the vials in a freezer (-15 to -25°C) for further usage (section 1C, step 9a, page 37).

  For tissue and some kind of fluid samples use *Enzyme K.* Replace the *Enzyme K* vial to the freezer (-15 to -25°C) immediately after handling.

  Caution: 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) Preparations for sample loading

For the following sample materials a pre-treatment with *Enzyme K* is essential for the usage in the instrument.

- Fluid samples:
  Mucous fluids, purulent fluids and fluids with flakes of tissue or solid particles (see part *i) Fluid samples*, method 2 of the transfer of the fluid sample).
- Tissue samples:
  Tissues, prosthesis, paraffin blocks, bones and other solid materials (see part *iii) Tissue samples*, page 25).

No enzymatic pre-treatment is necessary for clear or cloudy fluid samples (see part *i) Fluid samples*, method 1 of the transfer of the fluid sample) and swabs (see part *ii) Swabs*, page 25).

Do not use other specimens than validated in Tab. 6, page 19.

i) Fluid samples (ascites, BAL, cerebrospinal, EDTA or citrate-stabilised whole blood, joint aspirates, liver abscess puncture fluid, mucus, plasma, platelet concentrates, pleural fluid, pericardial effusion, synovial fluids, urine)

- Fluid specimens are sampled under aseptic conditions and transported to the laboratory.
- Transfer of the fluid specimens.
  - Method 1: Fluids without enzymatic pre-treatment

    Pipette 1 ml fresh fluid sample from the sample container into a *Plus-SV* vial (screw cap vials, Kit 1). If less sample volume is available, pipette the fluid into a *Plus-SV* vial and fill up to 1 ml (use the measure line of the tube) with buffer *SU* (Kit 1).

    Blood cultures (excluding cultures with activated carbon): Use 0.2 ml of the culture and fill up to 1 ml with buffer *SU*.
  - Method 2: Fluid samples with enzymatic pre-treatment

    Pipette 0.8 ml fresh fluid sample from the sample container into a *ST* tube (flip cap tubes, Kit 1). Add 180 µl of buffer *PKB* (Kit 1) and 20 µl of *Enzyme K* (Kit 2D) in the filled *ST* tube.

    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.

    After incubation, pipette the fluid phase into a *Plus-SV* vial (screw cap vials, Kit 1) by pipetting. **Avoid transferring any particles that may clog pipette tips and the columns in the instrument.**
    Comment: The particles in the fluid are partially digested and may decay. Potentially present bacteria and fungi are released.
- Transport the rack with the closed Plus-SV vials and *ET* tubes to the instrument. Continue with section 1C; page 26.

Caution: Gelatinous samples (e.g., sputum), cell cultures and blood cultures with activated carbon are inappropriate for the SelectNA™*plus*. This fluid samples may clog pipette tips and the columns in the instrument. Do not use these sample materials with the instrument.

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

- Do use only swabs without agar gel
- Pipette 1 ml of buffer *SU* (Kit 1) into a *ST* tube (flip cap tubes, 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).
- 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.
- Transfer the sample from the *ST* tube into a *Plus-SV* vial (screw cap vial, Kit 1). Transport the rack with the closed *Plus-SV* vials and *ET* tubes to the instrument. Continue with section 1C; page 26.

Caution: Swabs on agar gel media are inappropriate for the SelectNA™*plus*. This material in the swabs may clog pipette tips and the columns in the instrument. Do not use these sample materials with the instrument.

iii) Tissue samples (abscesses, aorta, artificial tissues, biopsies, bone marrow heart valves, lung tissues, mucous, pacemakers, paraffin blocks, pericard prosthesis)

- Tissue specimens are sampled under aseptic conditions and transported to the laboratory.
- Pipette 180 µl of buffer *PKB* (Kit 1) into a *ST* tube (flip cap tubes, Kit 1).
- 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. 0.5 x 0.5 x 0.5 cm. Cut the specimen into small pieces by using a sterile scalpel or sterile preparation scissors. Thereafter, transfer the dissected specimen to the *ST tube* filled with buffer *PKB.* The specimen should be covered completely by the buffer. Add 20 µl of *Enzyme K* (Kit 2D) to the specimen.
- 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.
  Comment: The tissue is partially digested and may decay. Potentially present bacteria and fungi are released.
- After incubation, pipette the fluid phase into a *Plus-SV* vial (screw cap vials, Kit 1) by pipetting.

  ! For this use the 200 µl pipette. **Avoid transferring any particles that may clog pipette tips and the columns in the instrument.**
- Fill up to 1 ml with the transport solution (**avoid transferring any tissue particles** from the transport solution), if available, or with buffer *TSB* (use the measure line of the tube). Transport the rack with the closed *Plus-SV* vials and *ET* tubes to the instrument. Continue with section 1C; page 26.

1C) Instrument Setup

Pathogen DNA is extracted and purified from clinical fluid samples, swabs and tissues in the ***SelectNA™plus*** instrument.

The Instrument

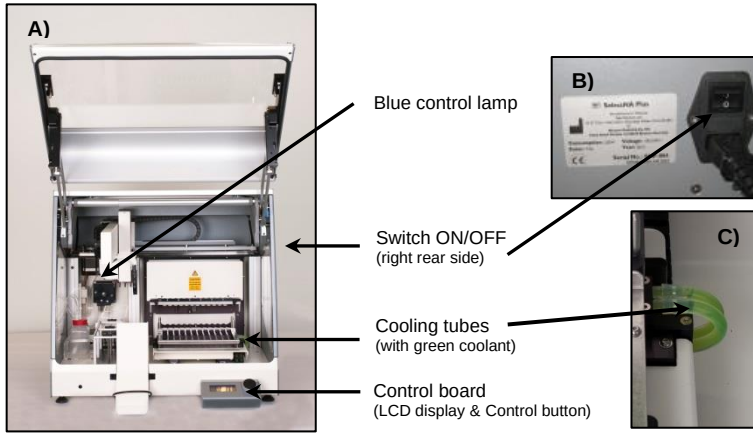


Fig. 3: The **SelectNA™*plus*** instrument.

Fig. 4: The interior of the **SelectNA™*plus*** instrument.

Pressure Monitoring System

The ***SelectNA™plus*** instrument includes a pressure monitoring system to reduce the risk of overflowing *Extraction columns*.

The system controls the *Extraction columns* after the filtration steps of the lysate. If residual liquid is detected on the column, the position is switched off and fluid is no longer transferred to this column position. The switched off positions will be indicated at the end of the extraction program in the display as “rejected channel” (details on page 28).

The pipetting arm is equipped with a sensor box including the pressure monitoring system (Fig. 5). The ***SelectNA™plus*** instrument possesses a four-channel pipette tip picking head for the uptake of up to four pipette tips at a time. For each channel a blue LED is located on the sensor box (Fig. 5).

Fig. 5: Pipetting arm with new pressure monitoring system.

Fig. 6: Four channels of the pressure monitoring system. The blue LED is switched on if the corresponding sample position of the pipetting block is blocked.

The blue LED is switched on if the corresponding column position is blocked or positions are not used during the pipetting process (e.g., 2 samples processed and LEDs of channels 3 and 4 switched on). The LED signals change for the next pipetting block. First pipetting block with samples 1 to 4, second block with samples 5 to 8 and third block with samples 9 to 12.

For example: Channel 1 is switched on in the first block (Fig. 6, part A). The column on sample position 1 is rejected. At the next block (samples 5 to 8) channel 3 is switched on (Fig. 6, part C) and the sample position 7 is rejected.

When the extraction program is finished, any rejected sample positions will be shown in the display, when applicable. It shows `Rejected channel: (position number)` and every position needs to be confirmed by pressing the `Control button` before the next position is displayed.

Example: Channel 3 is switched on in the first pipetting block and channel 4 in the third block. In the end it is summarized in the display with position 3 (Fig. 7, part A) and after confirming with the `Control button` with position 12 (Fig. 7, part B).

A)

B)

Fig. 7: Display shows the rejected positions at the end of the program. Example: A) The first rejected position 3 and B) the second rejected positon 12.

Loading Procedure of Components

Use Kits 1 (Cartridges & Consumables) and 2 (Enzymes).

Caution: Load the components direct in the instrument. Wear sterile protective gloves, sterile disposable sleeve covers, a disposable lab coat, protective goggles and a disposable mask when working in the instrument, loading the components and removing the *Elution tubes* after the extraction run (steps 2, 5 to 9, pages 30 to 40).

1. Initialisation and selection of the protocol

Fig. 8: Control board with Control button of the instrument.

- Turn ON the instrument on the right rear side of the instrument (Fig. 3B, page 26). The blue control lamp inside the instrument is on (Fig. 3, part A, page 26).
- Press the 'Control button' (Fig. 8) of the front control board to initialise the instrument.
- Press the 'Control button' to select the protocol from the 'Run Program Menu'.
- Turn 'Control button' to select script no. 1 'SelectNAplus' and press the button.

2. Waste bag

Place a *Waste bag* (not supplied, order no. D-928-500) to the waste chute and fix with the rubber ring (Fig. 9).

Caution: The exit of the waste chute must not be blocked by the waste bag, because otherwise pipette tips may accumulate in the chute and may fall into the interior of the instrument.

The screw caps of the enzyme and *Plus-SV* vials (step 9a and 9c, page 37) could be disposed to the waste bag.

Fig. 9: Waste chute with fixed *Waste bag*.

Load the following components (steps 3 and 5 to 9) direct in the instrument.

3. Reservoir with pipetting solution

Check that the reservoir is completely filled with pipetting solution (250 ml autoclaved deionized water;Fig. 10, on the left, and Fig. 4, page 26).

The tubings must be arranged on the left side of the reservoir (Fig. 10), because otherwise the tubes may block the pipette tip holder.

Confirm the loading step 'Load DI water bottle (250 ml DI water)' with the 'Control button'.

Fig. 10: The reservoir with pipetting solution.

4. Selection of the number of samples

- Select the number of samples to be processed. For this, turn the 'Control button' clockwise to select. The display shows the required number of full pipette tip rows, that are needed for the number of samples intended to be extracted. For further information on the tip rows see point 4 `Pipette tips`.
- Confirm 'Yes' and press the `Control button`. Select `No` to correct the number of samples. By selecting `No` you come back to the `Main Menu`.

5. Pipette tips

Note: Pipette tips for the instrument are not supplied with this kit. Use only Molzym´s DNA-free *Pipette tips* (order no. D-925-0xy) to avoid DNA contamination.

The pipette tip holder is loaded with two pipette tip racks. Each tip rack contains 96 tips that are arranged in 12 rows with 8 tips each (A1 to H1; see Fig. 11). The programming of the instrument follows an algorithm that is optimised for the usage of full tip rows remaining in the pipette tip holder after an extraction run. Tab. 7 shows the consumption of pipette tip rows for the needed number of samples. An example on page 32 is presented to explain the algorithm.

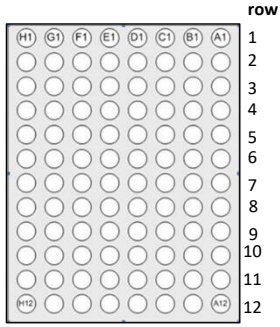


Fig. 11: Pipette tip rack

Tab. 7: Consumption of pipette tip rows dependent on the samples processed.

No. of samples	Tip rows used
1	4
2	8
3	8
4	8
5	10
6	12
7	16
8	16
9	18
10	20
11	23
12	23

Loading of the pipette tip holder:

Carefully load the pipette tip holder (Fig. 12, part 1) with pipette tip racks in the correct direction. Avoid touching the pipette tips on the tip holder. Fix the racks with the three black clips of the holder (Fig. 12, part 2 and 3).

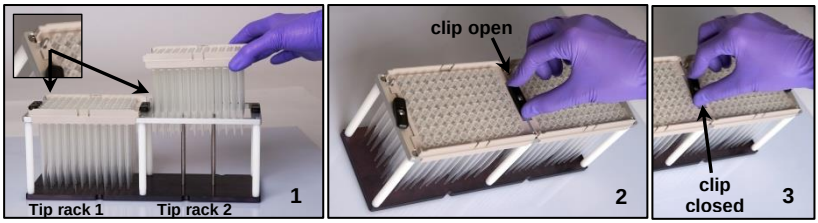


Fig. 12: Loading procedure of pipette tip racks into the tip holder.

Place the loaded pipette tip holder into the instrument (Fig. 13). Fit the notches of the holder in the 4 black knobs at the bottom of the instrument (Fig. 13, part 2 and 3).

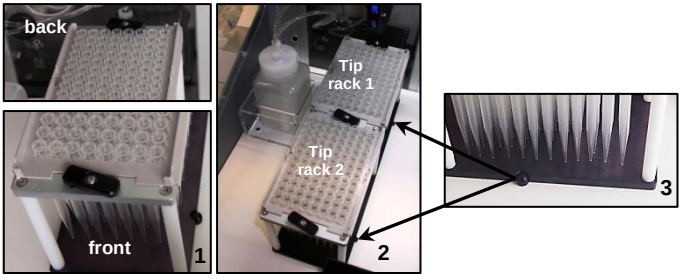


Fig. 13: Loading *Pipette tips* into the instrument.

Be sure that there are enough filled tip rows to run the selected number of samples (Tab. 7, page 30). The display shows the required number of rows. Select the tip row position (A1 to A12) and the tip rack (1 or 2). For the selection turn the 'Control button' clockwise.
Confirm this loading steps 'Load Pipette tip racks' and 'enter starting at full tip row' with 'Control button'.
Confirm `Yes` for the selected `starting tip full row` with the button.
Select 'No' to return to section 4 `Selection of the number of samples` (page 30).

Explanation of the algorithm for usage the pipette tip rows:

In this example, the instrument's pipette tip holder was initially loaded with two full pipette tip racks (2x 96 tips; see Fig. 13) and run with 7 samples which consumed 16 tip rows (Tab. 7, page 30). Accordingly, the tip rack 1 is empty and the tip rack 2 has 8 left over rows completely filled with tips (Fig. 14, page 32; tip rack 2 position row A5 to A12).

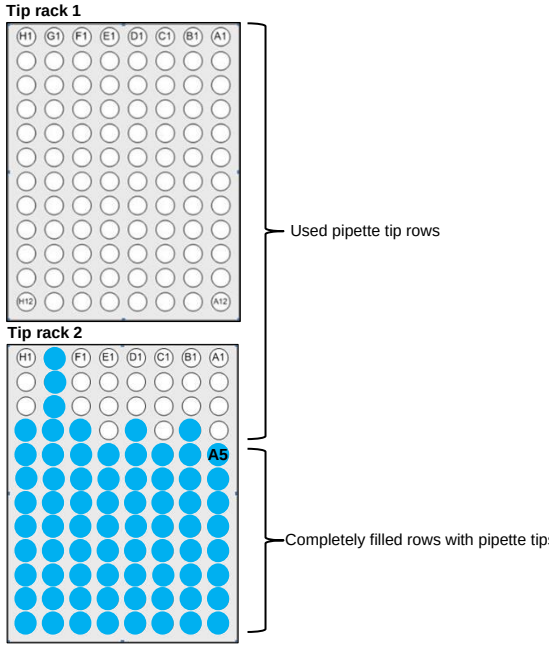


Fig. 14: Pipette tip racks at the end of running 7 samples. White positions are empty, blue positions filled with pipette tips.

Reference to Tab. 7 (page 30) tells one that up to 4 samples can be processed in a following run. Continuing with this example, if more than 4 samples (up to 10 samples) are desired to be processed, the following is recommended. Take care to avoid handling-borne contamination. Wear a disposable lab coat, protective gloves, arm sleeves and a disposable mask and follow the advices for avoidance of contamination (page 20).

Remove the empty tip rack 1 and place the partially filled tip rack 2 (Fig. 14) to the position of rack 1. Then place a new, full tip rack to tip rack position 2. Now, 8 rows (tip rack 1, position A5 to A12) plus 12 rows (tip rack 2), in total 20 rows are available to run up to 10 samples (Tab. 7, page 30). Continue loading the instrument with the other consumables and reagents and the sample as below.

For process 11 to 12 samples use two new full tip racks.

6. *Extraction columns*

Pick the columns with a sterile forceps and place them into the column rack from the left to the right side (Fig. 15).

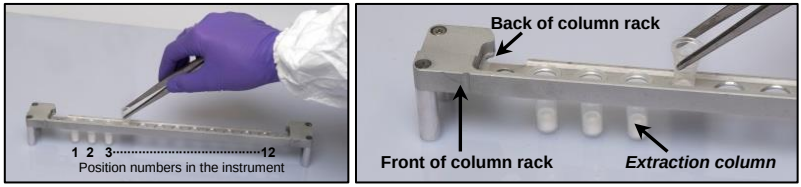


Fig. 15: Loading procedure of *Extraction columns* in the column rack.

Place the filled rack into the instrument in the black holder of the column rack. Push the column rack completely into the holder (Fig. 16, part 1).
Fix the rack with the black clips on each side of the holder (Fig. 16, parts 2 and 3).
Confirm this loading step 'Load Extraction columns' with 'Control button'.

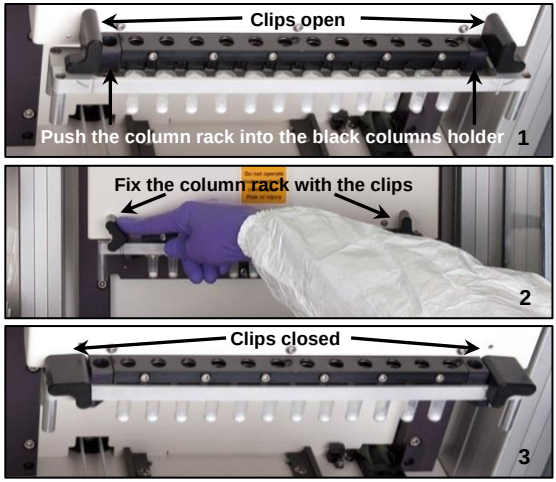


Fig. 16: Loading procedure of the filled column rack into the instrument.

7. *Extraction cartridges*

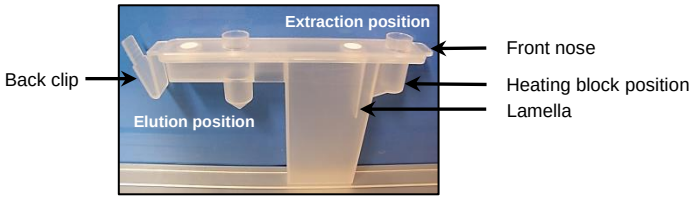


Fig. 17: *Extraction cartridge*

Load the extraction rack with the *Extraction cartridges* (Fig. 17), starting from the left to the right side (position numbers 1 to 12, Fig. 18, part 1).
Place the front nose of the *Extraction cartridge* in a slanted angle under the metal edge (Fig. 18, part 1).
Push the *Extraction cartridge* into the rack until the back clip locks in position with a click sound (Fig. 18, part 2).

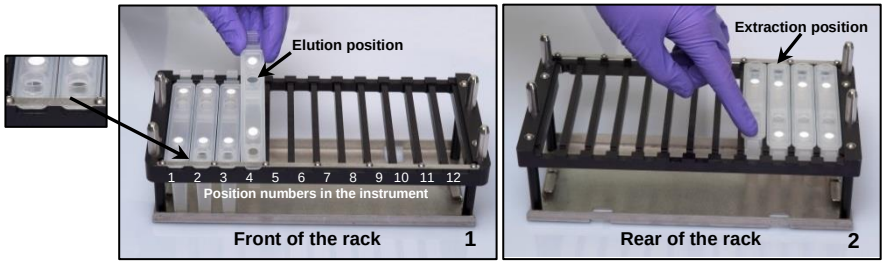


Fig. 18: Loading procedure of *Extraction cartridges* into the extraction rack.

Place the rack into the instrument. Take care that cartridges are placed with the extraction position in the corresponding indentation in the heating block. Fix the rack with the black clips on both sides (Fig. 19).
Confirm this loading step ‘Load vacuum cartridges’ with ‘Control button’.

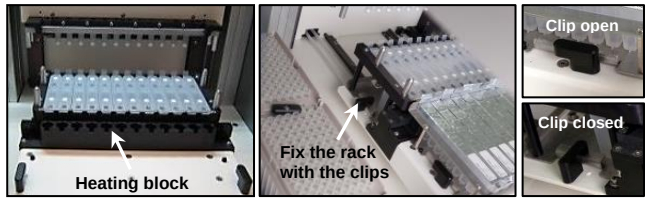


Fig. 19: Loading procedure of the filled *Extraction cartridge* rack into the instrument.

8. *Buffer cartridges*

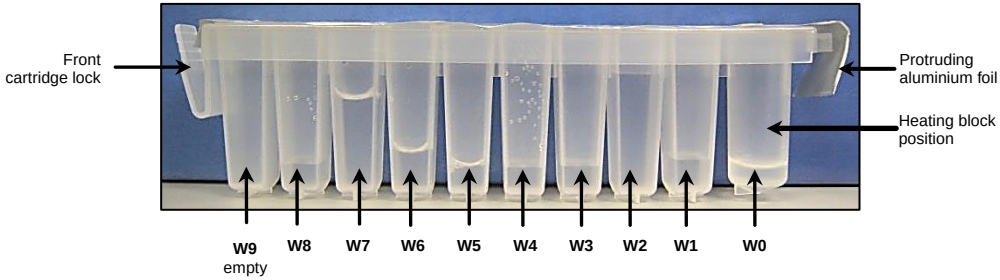


Fig. 20: Filled Buffer cartridge (DNA-free).

Bend up the protruding aluminium foil at the rear of the *Buffer cartridge* and load the buffer rack with the *Buffer cartridges* (Fig. 20). Place the front nose of the *Buffer cartridge* in a slanted angle under the metal edge (Fig. 21, part 1). Push the *Buffer cartridge* in the rack until the back clip locks in position with a click sound (Fig. 21, part 2).

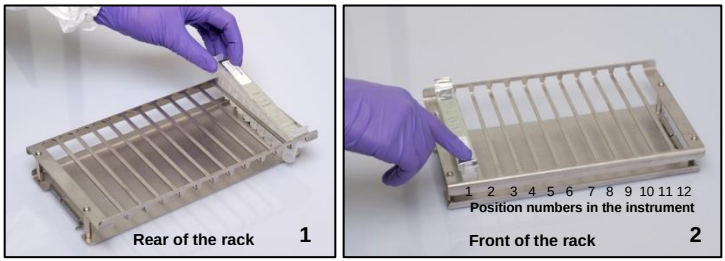


Fig. 21: Loading procedure of *Buffer cartridges* into the rack.

Place the rack into the instrument. Take care that the back position of the cartridge (round W0) is placed into the corresponding indentation in the heating block (Fig. 22). Fix the rack with the black clips on both sides (Fig. 22).

Fig. 22: Loading procedure of the filled buffer rack into the instrument.

Carefully peel off the aluminium foil by pulling constantly and slightly directed to the side (Fig. 23, parts 1 and 2). Do not touch the reagent wells. At the end, check that the cartridges are fixed flat in the rack.
Confirm this loading step 'Load Buffer cartridges and peel off aluminium foil' with the 'Control button'.

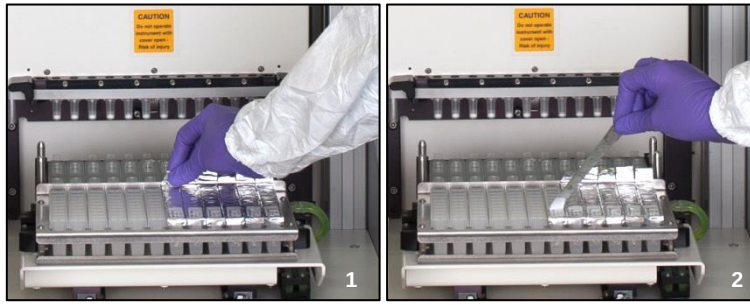


Fig. 23: Removing of the aluminium foil of the *Buffer cartridges*.

9. Loading of Reagent vial rack

Close the door and press 'Control button' for loading the Reagent vial rack.

The display on the Control board reads:
'**WARNING** Transferring rack. Press button'

Press the button again and the rack with the cartridges moves backward. **Caution:** Keep your hands off the instrument!

After movement of the cartridge rack, open the door. Place the Reagent vial rack into the instrument.

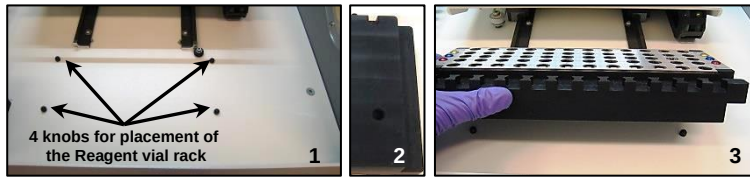


Fig. 24: Placing Reagent vial rack in the instrument.

For this, place the notches on the bottom of the rack (Fig. 24, part 2) into the 4 black knobs at the bottom of the instrument (Fig. 24, part 1). For this, slide the rear bottom notches of the rack in the rear knobs (Fig. 24, part 3) and then place the front bottom notches in the corresponding knobs.

Place the following vials in the sequential steps (parts 9a to 9c, next page) in the Reagent vial rack of the instrument (Fig. 25, page 37). Place the vials from left to right. Each row of enzyme vials is marked with a coloured knob at the left and right side of the Reagent vial rack.

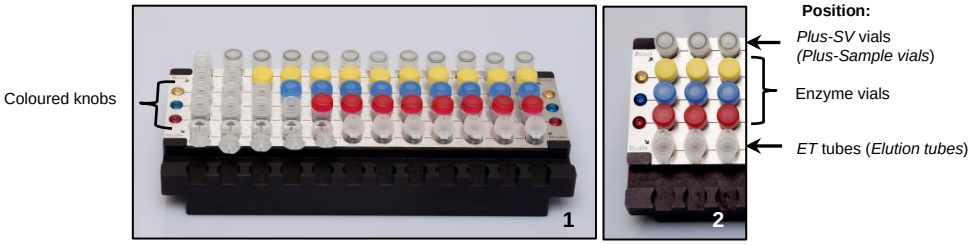


Fig. 25: Reagent vial rack

9a) Enzymes and colour code

- *BugLysis plus*, yellow cap
- *Proteinase K*, blue cap
- *MolDNase C*, red cap

! Remove the rack with enzyme vials from the freezer (section 1A, page 23). Make sure that the enzyme vials have been pulse centrifuged to clear the lids.

At first, open the *BugLysis plus* vials (yellow cap) and place them into the Reagent vial rack at the position in row with the yellow coloured knob, following positions 1 to 12 in the Reagent vial rack from left to right (Fig. 25). Dispose the screw caps to the waste bag.

Caution: *BugLysis plus* contains 2-mercaptoethanol which is toxic. Take care not to inhale and otherwise come into contact with.

Secondly, open the *Proteinase K* vials (blue cap) and place them into the Reagent vial rack at the position in row with the blue coloured knob, following positions 1 to 12 in the Reagent vial rack from left to right (Fig. 25). Dispose the screw caps to the waste bag. Confirm the loading step 'Load reagents rack, Enzymes blue cap' with 'Control button'.

Lastly, open the *MolDNase C* vials (red cap) and place them into the Reagent vial rack at the position in row with the red coloured knob, following positions 1 to 12 in the Reagent vial rack from left to right (Fig. 25). Dispose the screw caps to the waste bag.

9b) *Elution tubes* (*ET* tubes)

Open lids of the Elution tubes, marked with the sample ID (section A, page 23). Place the opened *Elution tubes* to the elution position following positions no. 1 to12 of the Reagent vial rack (left to right; Fig. 25). Adjust the lids of the tubes to the front of the rack (part 1; Fig. 25).

9c) *Plus-Sample vials* (*Plus-SV* vials)

Remove the screw cap from each *Plus-SV* vial (see part B, page 24) containing the sample and place into the Reagent vial rack following positions 1 to 12 (left to right; Fig. 25). Dispose the screw caps to the waste bag.

9d) ***Safety cover***

After loading of the Reagent vial rack with enzymes, *Elution tubes* and the *Plus-SV* vials place the Safety cover on the rack, if available for the instrument (Fig. 26).

Fig. 26: Safety cover for the Reagent vial rack.

Confirm the loading step ‘Load reagents rack ` with ‘Control button’.

Check that the aluminium foils of the *Buffer cartridges* have been removed.

Check, that all vials stand correctly at the same level in the rack and all caps have been removed. Confirm this step `Check all caps removed` with the ‘Control button’.

Close the door of the instrument.

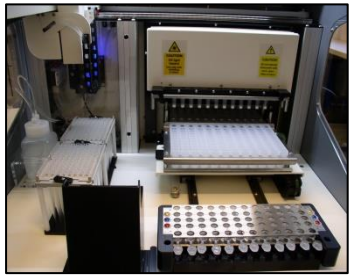


Fig. 27: Loaded instrument.

10. Waste chute and bag

Check that a waste bag was fixed at the waste chute and the exit of the chute is not blocked with the bag. Otherwise pipette tips may accumulate in the chute and may fall into the interior of the instrument. Further information see step 2, page 29.

Confirm the loading step ‘Load waste chute and bag’ with the ‘Control button’.

11. Start the extraction process

Close the door of the instrument and confirm this step ‘Close door and press button to start’ with ‘Control button’. The instrument now starts the extraction.

The program is finished with a signal sound. The approx. total time of the program for the corresponding sample number see in Tab. 8.

Tab. 8: Total time of the extraction process.

No. of samples	Approx. total time [min]
1 to 4	95
5 to 8	108
9 to 12	126

Caution: Wear sterile protective gloves, sterile disposable sleeve covers, a disposable lab coat, protective goggles and a disposable mask when opening the door for closing and removing the *Elution tubes* from the instrument.

Open the door and remove the Safety cover of the Reagent vial rack (if available). Close the *Elution tubes* including the eluate and remove them from the instrument. Confirm this step ‘Open door’ with the ‘Control button’.

Any samples rejected by the Pressure Monitoring System (pages 27 to 28) will be shown in the display, when applicable. Every rejected position needs to be confirmed by pressing the ‘Control button’.

12. Eluted DNA

Store the eluted DNA at +4 to +12°C if analysed within 48 hours or freeze at -15 to -25°C for longer storage. Avoid frequent freeze-thaw cycles, because this may result in loss of eluted DNA (in particular at low DNA concentrations).

Volume of the eluate:
The mean eluate volume amounts to 90 µl (range approx. 70 to 140 µl). A volume <70 µl is possible and does not have influence on the result.
If volumes are higher, this may indicate a malfunction of the drying of the membrane which leads to elution of ethanol. This in turn would inhibit the PCR reaction. In this case the extraction should be repeated.

Decontamination of the Instrument

You need a low-lint, soft paper towel for the cleaning procedure. For disinfection employ Meliseptol

® New Formula (B. Braun, Germany) or an ethanol containing disinfectant. After use, dispose the paper towel in the waste for infectious material. Do not spray surfaces inside the instrument with the disinfectant. Instead use a paper towel sprayed with disinfectant and wipe the surfaces.

1D) Decontamination after each run

1. Dispose the empty *Plus-SV vials* and enzyme vials to the waste bag (use the waste chute). **Caution**: 2-mercaptoethanol is a toxic compound included in *BugLysis plus* vial (yellow capped). Take care not to inhale and otherwise come into contact with when removing the vial.
2. Decontamination of the waste chute: Spray the contaminated surfaces with the disinfectant and wait for 10 min.
3. Remove the Reagent vial rack. Clean the rack and his Safety cover. Wipe the surface of the instrument around the rack with a paper towel soaked with disinfectant. Replace the rack back to the instrument.
4. The display of the Control board indicates: '**WARNING** Transferring rack. Press button'. Close the door.

   Press the button and the vacuum rack with the cartridges transfers forward. **Caution:** Keep your hands off the instrument!
5. Remove the waste bag and dispose the used *Buffer cartridges*, *Extraction cartridges* and *Extraction columns* to the waste bag. For this, open the clips of the racks and take out the racks with the used consumables. At the end, dispose the waste bag to the waste for infectious material.

   Caution: Do not take out single cartridges (*Buffer* and *Extraction cartridges*) from the racks while the racks are fixed in the instrument. This may damage the racks and heating blocks.

   Note: In case of a contaminated filter of the *Extraction cartridge* (extraction position) with liquid from the waste, clean the corresponding suction cup of the vacuum system. For cleaning see section 1F, page 42.
6. Storage partially filled tip racks in the pipette tip holder in the instrument. The completely filled tip rows in rack 1 or 2 can be used for the following run. Remove empty tip racks from the pipette tip holder.
   If applicable, remove the tip rack 1 from the pipette tip holder. Place the partially filled tip rack 2 to position of rack 1 of the pipette tip holder.

   Note: Take care to avoid handling-borne contamination and follow the advices for avoidance of contamination (manual on page 20).
   Do not use the remaining pipette tips in the used rows and do not re-assemble the tips in the pipetting tip racks to avoid contamination of following extractions.
7. Clean the removed racks. For this wet the racks completely with disinfectant and wait for 5 min. After the time of exposure wipe the racks with a paper towel soaked with the disinfectant and let dry the racks on air before replace into the instrument.
   Clean the suction cups and the surfaces of the instrument with a paper towel soaked with disinfectant.

   Do not spray the interior of the instrument! **Caution:** Do not clean the pipetting arm, the control board, the chains, cooling tubes and the windows of the

instrument with the disinfectant.

8. Replace all racks to the instrument. Place the column rack to the right side of the pipette tip holder for UV cleaning (Fig. 28, part 1).

   Place the cleaned safety cover of the reagent vial rack on the right side of the instrument. The back side of the cover is face in the inside of the instrument (Fig. 28, part 1 and 2). The cover is place on the black feet (Fig. 28, part 2).

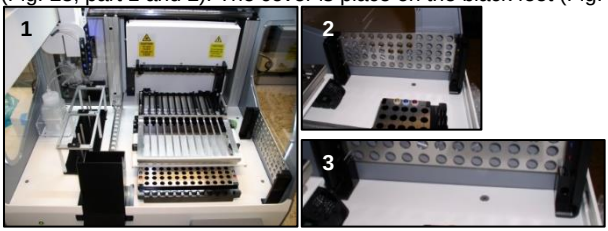


Fig. 28: Interior of the instrument for UV decontamination of the column rack and safety cover.

9. Check that minimum 100 ml pipetting solution (autoclaved deionized water) is still in the reservoir. If not, fill the reservoir completely (max. 250 ml) with autoclaved deionised water (Fig. 4, page 26).

   Press the 'Control button' to finalise the protocol.

10. Close the door. Wipe the door handle and the door top with a paper towel soaked with disinfectant and select the 'UV decontamination' from the 'Main Menu'. Confirm the program with the 'Control button'. Confirm the loading steps of the empty racks with the 'Control button'. The instrument now starts the UV decontamination. The program is finished with a signal sound. Open the door and confirm this with the 'Control button'.

    ! Decontamination (steps 1 to 6, section 1D) and UV decontamination should be performed after each run of the instrument. The instrument must be empty before starting the decontamination program.

11. Start a new extraction run or continue with section 1E 'Daily decontamination of the instrument'.

1E) Daily decontamination of the Instrument

At the end of each day of usage clean the waste chute.

1. Remove the decontaminated waste chute (section 1D 'Decontamination of the instrument after every extraction run') from the instrument and clean it.
2. Clean the chute in a special washing machine using a mildly alkaline cleaning powder with sodium hydroxide (e.g. LABWASH® Premium Classic, VWR). Dry the clean chute with a low-lint, soft paper towel.

   Optionally, put the waste chute in a washing solution (e.g., LABWASH® Premium Classic, VWR). The waste chute must be completely covered by the washing solution. Incubate the waste chute as described in the instruction and rinse the chute with water. Dry it with a low-lint, soft paper towel.
3. Thereafter place the clean chute back to the instrument
4. Close the door and select script 2 'UV decontamination' from the 'Run Program Menu'. Confirm the step 'Start decontamination' with the 'Control button'. The instrument now starts the UV decontamination. After 5min the program is finished with signal sounds. Confirm this with the 'Control button'.

   ! The instrument must be empty before starting the decontamination program.

1F) Cleaning script - Cleaning of the pipetting system

Clean the pipetting system every 14 days of usage.

1. Supplied material to be used: 4 *Cleaning cartridges* (Fig. 29; supplied with the instrument, Molzym order no. D-927-012) and *Cleaning bottle* (Fig. 29).

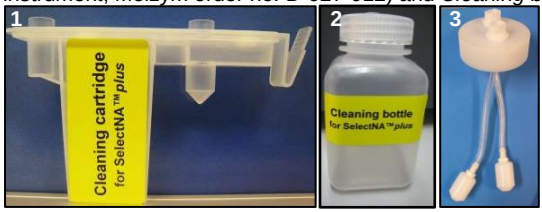


Fig. 29: *Cleaning cartridge* (1), *Cleaning bottle* (2) and lid (3).

Prepare a bleach solution (1 % (active Cl_2) sodium hypochlorite): For this, mix 14.3 ml of sodium hypochlorite (14 % active Cl_2, VWR Chemicals) and 185.7 ml autoclaved deionized water. Fill the *Cleaning bottle* with 100 ml prepared bleach solution.

2. Start the cleaning script from the 'Cleaning Menu' for cleaning the pipetting tubes.
3. Load the four *Cleaning cartridges* (Fig. 29, page 42) into the rack of the *Extraction cartridges* (positions 1 to 4), place the rack into the instrument and fix on both sides with the black clips (see Fig. 18 and Fig. 19, page 34). Confirm the step with the 'Control button'.
4. Remove the reservoir with the pipetting solution incl. the lid (Fig. 10, page 29) from the instrument. The tubing are fixed with a click-system on the lid. Connect the filled *Cleaning bottle* with the second lid for the pipetting tubes. Confirm the step 'Load cleaning bottle (100 ml 1 % bleach)' with the 'Control button'.
5. Close the door of the instrument and start the cleaning procedure by pressing the 'Control button'. The tubing incubates 10 min with the 1 % bleach solution.
6. Empty the reservoir bottle and clean with 1 % bleach. For this, fill the bottle with the bleach solution (~50 ml), close it with the screw cap of the *Cleaning bottle* and shake it. Empty the bottle and rinse with autoclaved deionized water.

   Clean the lid of the reservoir bottle with a low-lint, soft paper towel soaked with the 1 % bleach solution. After this, rinse the lid with autoclaved deionized water and dry it with a paper towel or on air. Fill reservoir with 250 ml autoclaved deionized water and close it with the cleaned lid.
7. After the 'soaking (wash)' step (10 min) indicates with a signal sound, open the door and remove the *Cleaning bottle* incl. the lid. Connect the cleaned reservoir filled with autoclaved water on the tubing. Close the door of the instrument and resume the program by pressing the 'Control button' to rinse the pipetting tubes.
8. Remove the lid from the *Cleaning bottle*, close it with the screw cap and shake it. Empty the bottle and rinse with autoclaved deionized water. Clean the lid (with the tubing) of the *Cleaning bottle* as per description of the lid in step 6.
9. At the end of the cleaning program, remove the cartridge rack from the instrument. **Caution:** Do not take out the *Cleaning cartridges* from the rack while fixed in the instrument. This may damage the racks and heating block.

10. Dispose the solution from the *Cleaning cartridges* and rinse with water. Dry the *Cleaning cartridges* on air and use for the next cleaning procedure.
11. Clean the cartridge rack with a paper towel soaked with disinfectant. Replace the empty rack into the instrument.
12. Close the door of the instrument, select the 'UV decontamination' (Version V.0.04) from the 'Main Menu' and start the UV decontamination program. After 5 min the program is finished indicated by a signal sound. Shortly lift the door to close the signal sound.

Caution: Dispose waste including sodium hypochlorite in accordance with federal, state and local regulations. Avoid runoff into storm sewers and ditches which lead to waterways (concentration active chlorine >0.25 %).

Dispose bleach solution separately to the *Extraction cartridges* and Enzyme vials, because of risk of cyanide formation.

1G) Cleaning of the vacuum system

In a case of an overflowed sample clean the white suction cups of the vacuum system, the black holder of the column rack (Fig. 30) and the column rack by wiping. Do not spray the interior of the instrument!

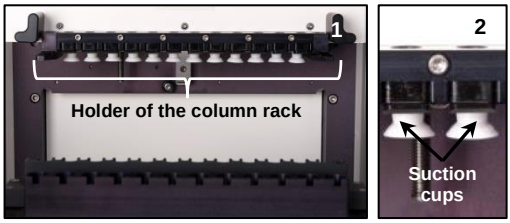


Fig. 30: Holder of the column rack (1) and suction cups (2) of the vacuum system.

For this, pull the suction cups from the system and clean with a paper towel soaked with disinfectant (Fig. 31). Push the dry suction cups back to their position in the column holder.

Fig. 31: Cleaning of the suction cups.

For further information see `Troubleshooting` on page 65.

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)*
  – Detection Reagents & Positive PCR Control
- ***Consumables PCR Detection & Sequencing (Kit 1)*** *(store at +18 to +25°C):*
  – MT - Mastermix tubes, 1.5 ml

Part 2 – Analytics

Description of the Assays

With this unit, PCR assays are supplied for the testing of the presence of bacterial and fungal DNA in clinical specimens. The 'Mastermix Assay Bacteria' (*MA Bac*, Kit 3) and 'Mastermix 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 mastermixes *MA Bac* and *MA Yeasts* (Kit 3) for the PCR amplification of sequences using extracted DNA (pages 21 to 41) 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 54 to 62).
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 'Mastermix 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 the samples. The correct function of the extraction process and the absence of PCR inhibitors potentially co-eluted with the target pathogen DNA are monitored by *MA Control*.

Protocols for amplification are supplied for the following instruments:

- Thermal Cycler (protocol, page 50):
  - Mastercycler® Eppendorf
- Real-Time PCR instruments (addendum pages 55 to 58):
  - LightCycler® 1.5, 2.0, 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 *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 46 to 52).

Packaging, Storage and Handling

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

The purification and packaging of the mastermixes supplied in this PCR detection unit are performed under standard precautions for the avoidance of air-borne and handling-based DNA contaminations. The mastermixes are supplied as a 2.5x concentrated solution in DNA-free screw cap vials (Kit 3). For usage, the mastermixes are thawed at room temperature (+18 to +25°C). After use, the mastermixes 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 has to be kept at -15 to -25°C throughout handling (cooling rack). Replace *MolTaq 16S/18S* to the freezer (-15 to -25°C) after handling. **Caution:** 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.
It is important to note that the DNA staining solution (*DS*, Kit 3) and DNA size marker (*SM*, Kit 4A) are sensitive to light and should be stored in the dark during handling and storage. **Once thawed, do not freeze again** and store at +4 to +12°C for further use.

Store the *Gel loading solution* (*LS*, Kit 4A) after first handling at +4 to +12°C.
Store *DNA dilution buffer* and *DNA Standard P1* (Kit 4B) at +4 to +12°C after thawing.

Take care to maintain a DNA-free environment during opening the vials and handling the mastermixes by working under a UV-decontaminated workstation. Use only certified microbial DNA-free pipette tips and PCR consumables recommended for running the assays (pages 7 to 8).

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*.

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

PCR Detection and Identification of Pathogens by Sequencing

PCR Detection

Assay *MA Bac* and *MA Yeasts*:
With each mastermix an extra volume is supplied to run PCR controls. Negative PCR controls should always be performed, at least with 10 % of the sample runs, to test for potential cross-contamination or other carryovers resulting from handling or air-borne errors. Also, with each set of sample runs positive PCR controls (page 16) must be included to control the performance of the assay. Follow the instructions for the performance of positive PCR controls (pages 47 to 50).
Assay *MA Control*:
The Assay *MA Control* (Internal Extraction Control; Kit 3) is a test to check the performance of the extraction process and to confirm the absence of co-eluted PCR inhibitors together with the sample DNA. *MA Control* has to be performed with each sample testing (pages 47 to 50).
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 positive 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.

Exceptional cases:
If the sample lacks a PCR signal, the Internal Extraction Control is positive and 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. In this case, all PCR controls must perform as expected.

2A) How to Start

! For equipment, consumables and reagents to be supplied by the user see pages 7 to 8.

Avoidance of DNA Contamination

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

For more details, see page 20.

Storage of the PCR Reagents after Handling

! After use, keep the 2.5x mastermixes and H_2O in a refrigerator (+4 to +12°C) if reused at the same day or store at -15 to -25°C for longer periods
! Replace *MolTaq 16S/18S* in a cooling rack (-15 to -25°C). Always keep and store *MolTaq 16S/18S* at -15 to -25°C. **Do not interrupt the cooling of *MolTaq 16S/18S*.**
! After first use, store *DNA staining solution* (*DS*) in the dark at +4 to +12°C. **Do not re-freeze**.
! After the first use, store *DNA dilution buffer* and *DNA Standard P1* at +4 to +12°C.
! Diluted DNA solutions (P2) tend to be unstable. Always prepare P2 freshly for each series of PCRs (step 8, page 50).

For more information about the storage and stability of the PCR reagents see Tab. 1, page 5.

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 assays
- Positive PCR controls P1 and P2

PCR Assays

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

DNA-free

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

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

MA Control (*Internal Extraction Control*, IEC)
- 1 reaction per sample
- 1 reaction for negative control (NC IEC)

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

DNA-free

Kit 3:
- *H_2O*
- 2.5x *MA Bac*
- 2.5x *MA Yeasts*
- 2.5x *MA Control*
- *DS;* keep dark

DNA

Kit 4B:
- *DNA Standard P1*
- *DNA dilution buffer* (for P1)

Before starting with the preparation of PCR-ready mastermixes (section 2B, page 49):

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

2B) Setup of the Assays

Keep all PCR tubes filled with PCR-ready mastermix and *MolTaq 16S/18S* 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 PCR-ready mastermixes *MA Bac*, *MA Yeasts* and *MA Control*

1. **Arrange the PCR tubes for *MA Bac*, *MA Yeasts* and *MA Control* in a PCR cooling rack (-15 to -25°C) and mark (PCR Assaying, page 48).**
2. **Place *MolTaq 16S/18S* (Kit 3) 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 (Tab. 9) into each *MT* tube. Vortex the tubes to mix and briefly centrifuge.
4. **Pipette 20 µl of the PCR-ready mastermix *MA Bac* into each PCR (dedicated for samples, P1, P2 and NC, respectively). Repeat the procedure with mastermix *MA Yeasts*. Pipette 20 µl of the PCR-ready mastermix *MA Control* into each PCR tube (dedicated for samples and NC).**
5. **Add 5 µl H_2O (DNA-free water; Kit 3) into the NC PCR tubes. Close all PCR tubes with the caps.**
6. **Place the PCR tubes in another cooling rack designated for transport to the UV Class II biological safety cabinet.**

DNA handling

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

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

Positive PCR controls *P1* and *P2*

8. **Vortex the *P1* vial and pulse centrifuge. Pipette 998 µl of *DNA dilution buffer* into a 1.5 ml sterile polypropylene tube (not supplied). Add 2 µl *DNA Standard P1* and vortex to mix (*P2*). Briefly centrifuge.**
9. **Pipette 5 µl of positive PCR control *P2* into a PCR tube containing *MA Bac* and *MA Yeasts*, respectively. Repeat with *P1*. Close PCR tubes with caps.**
10. **Continue with section 2C PCR Thermocycling page 50.**

Tab. 9: Preparation of PCR-ready 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) prepared as above to a place where PCR runs are performed. Programme the Mastercycler® (Eppendorf) as described (Tab. 10). After the PCR run go to section 2D for the detection of amplicons.

Tab. 10: PCR programme for Mastercycler® (Eppendorf)

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

2D) Detection by Agarose Gel Electrophoresis

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

! For equipment, consumables and reagents to be supplied by the user (pages 7 to 8).

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

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).

- Per PCR reaction, pipette 8 µl of the PCR product into a sterile 1.5 ml polypropylene tube (not supplied) or in a well of a 96 well plate (not supplied) and mix with 2 µl of the gel loading solution (*LS*). Mix by pipetting in and out for several times.
- 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 *SM* (DNA size marker; Kit 4A).
- Close the electrophoresis chamber with the cover and run the gel at 10V/cm interelectrode distance in the dark.
- Leave the gel running until the blue dye has moved about 2/3 of the way through the gel. At the conditions described this takes about 30 to 45 min.
- 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 Fig. 32 and Fig. 33, pages 51 to 52.
  Note: If all bands showed a too weak fluorescence signal, the gel can be re-coloured in e.g. an ethidium bromide staining bath, if necessary.
- Make sure that bands appear with in the samples in the assay *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 Fig. 32. In this case, samples were collected from six patients and processed as described in the previous sections.
The controls were as expected, i.e. positive PCR controls P1 and P2 showed bands at the correct position (approx. 450bp, see 'bacterial DNA' in Fig. 32).
The negative PCR controls (NC) did not show a signal, so DNA contamination can be excluded.
The Internal Extraction Controls C1 to C5 of the samples 1 through 5 (Fig. 32) showed clearly visible bands (approx. 200bp), 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.
Five of the samples showed bands (samples 1 to 3, 5, 6) in the assay *MA Bac* at the expected gel position (Fig. 32) and thus were positive for bacterial DNA. Sample 4 (Fig. 32) was negative (only showing weak bands at approx. 320bp, indicating traces of amplified human DNA; 'human DNA', Fig. 32).

Banding of amplification products from Assay *MA Yeasts* (fungi) appear at a position of approx. 310bp (Fig. 33, page 52).

Exceptional cases:
In the case of the specific bacterial band in the sample test (6, Fig. 32) but lacking signal in the Internal Extraction Control (C6), the positive PCR sample result is valid.

All positive PCR samples have to be identified by sequencing.

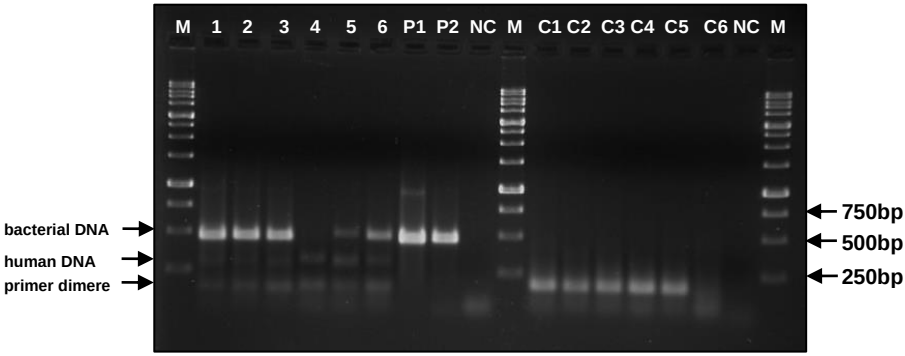


Fig. 32: PCR analysis of eluates from 6 patient samples (1 to 6) using Mastermix Assay Bacteria (*MA Bac*). *P1, P2*: Positive PCR controls; NC: Negative PCR controls; C1 through C6: Internal Extraction Controls (*MA Control*) with respective eluates from patient samples 1 to 5 (6: negative) (banding at approx. 200bp); M: DNA size marker (*SM*). The weak unspecific signals (left side of the figure; arrow 'human DNA', approx. 320bp) below the specific signals from samples (arrow 'bacterial DNA', approx. 450bp) are the result of the amplification of traces of human DNA, well separated from the specific bands.

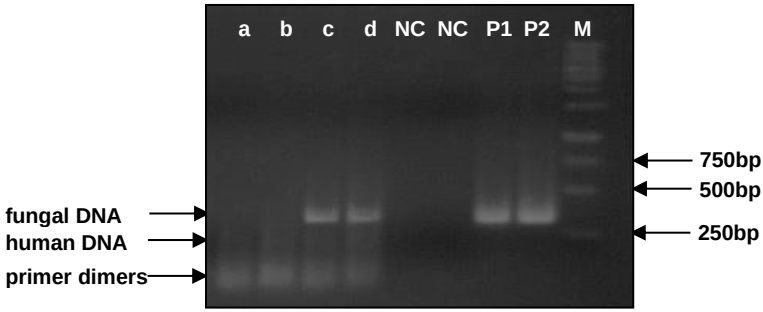


Fig. 33: PCR analysis of eluates from four patient samples (a to d) using Mastermix Assay Yeasts (*MA Yeasts*, fungi). P1, P2: Positive PCR controls; NC: Negative PCR control; M: DNA size marker (SM). Signals at the position 'fungal DNA' (310bp) indicate that samples of patients c and d contain fungal DNA (sequencing result: *Candida albicans*). The weak signals at approximately 250bp (arrow 'human DNA') are the results of the amplification of traces of human DNA.

Identification of Pathogens by Sequencing of Amplicons

All positive PCR samples have to be identified by sequencing.
Sequencing of amplicons together with BLAST online homology search is used for the identification of pathogens detected by ***Micro-Dx™***. Sequence analysis has been validated with ***Micro-Dx™***. 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. (http://www.sepsitest-blast.com). For further investigations see section 2H, page 53.

2F) Purification of Amplicons

For sequencing, amplicons need to be purified. Qiagen's QIAquick® PCR Purification Kit (cat. no. 28104) has been validated with ***Micro-Dx™***. 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 54 to 62) 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, page 53).

2G) Sequencing

Apply the purified amplicon DNA to a sequencing reaction as advised by the manufacturer of the sequencing system. ***Micro-Dx™*** has been validated using Applied Biosystems DNA Analyzer ABI 3730XL® and ABI Prism310® apparatuses together with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). For sequencing, use the sequencing primers (10µM each) supplied with Kit 4. For sequencing of amplicons 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 detect by *SeqGP16*. *SeqGP16* targets mainly Gram-positive bacteria, including few gram-negative species. The excluding species will be detect by *SeqGN16*.
The exceptions of the sequencing primers see in table 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) SepsiTest™-BLAST Analysis for Strain Identification

Molzym has developed a free online service (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 characterised by a pool of more than 7,000 quality-controlled complete sequences of the 16S and 18S rRNA genes of only cultured and denominated eubacteria, *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 (http://www.sepsitest-blast.com/en/faqs.html).
Please note: For comparison, we recommend to blast sequence results also with another tool e.g., NCBI BLAST http://www.ncbi.nlm.nih.gov/blast. The result with the best score is valid.
Note: Sequence identities ≥97 to <99 % should be interpreted as on the genus level, ≥99 % as on the species level (Wellinghausen et al. 2009). Sequence identities below 97 % are rejected by SepsiTest™-BLAST. This may be the result of reading errors of the sequencing reaction. In such a case it is recommended to inspect the densitogram read-out for overlying sequences indicating the presence of more than one strain in the sample (Fig. 2, page 15). Overlying sequences can be resolved using a specialised tool, RipSeq® (Pathogenomix; http://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 ***Micro-Dx™*** 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® 1.5, 2.0, 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 58 to 62) 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 44 to 52). 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 46 to 50).

! Calibrate your Real-Time PCR machine using the Assays *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 47 to 50. The specific thermocycling conditions are described on pages 55 to 57. For the preparation of mastermixes, follow the instructions (part 2, sections 2A to 2C, pages 47 to 50). 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 58).

! For equipment, consumables and reagents to be supplied by the user see pages 7 to 8. In addition, the following items are needed to perform Real-Time PCR:

- 1x Real-Time PCR machine (above). Other instruments have to be validated for their use.
- PCR tubes e.g., glass capillaries (20 µl) for Lightcycler®1.5 (25 µl final volume per assay) or PCR strips (8x 0.2 ml) for other 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.

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

Real-Time PCR Thermocycling and Detection by Melting Curve Analysis

A melting curve analysis has always to be performed in order to discriminate possible primer dimer formation from true pathogen signals. See examples on pages 57 to 62. All positive Real-Time PCR sample results have to be identifying by sequencing. For sequencing of amplicons see part 2, section 2F to 2H, pages 52 to 53.

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

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

Method	Cycles	Analysis Mode	Target Temperature [°C]	Incubation time [hh:mm:ss]	Ramp rate [°C/s]	Acquisition [per °C]	Acquisiton Mode
Initial denaturation	1	None	95	00:01:00	4.00	-	NONE
Cycling	40	Quantification	95	00:00:05	4.00	-	NONE
			55	00:00:05	4.00	-	NONE
			72	00:00:25	4.00	-	SINGLE
Melting	1	Melting Curve	95	00:00:00	20.00	-	NONE
			65	00:00:15	20.00	-	NONE
			95	00:00:00	0.05	-	CONT
Cooling	1	None	40	00:00:05	20.00	-	NONE

II) Roche LightCycler® 96

Method	Cycles	Analysis Mode	Target Temperature [°C]	Incubation time [hh:mm:ss]	Ramp rate [°C/s]	Acquisition [per °C]	Acquisiton Mode
Initial denaturation	1	None	95	00:01:00	4.40	-	None
Cycling	40	Quantification	95	00:00:05	4.40	-	None
			55	00:00:10	2.20	-	None
			72	00:00:25	4.40	-	Single
Melting	1	Melting Curve	95	00:00:01	4.40	-	None
			70	00:00:01	2.20	-	None
			95	-	0.2	5	Continuous
Cooling	1	None	40	00:00:10	-	-	-

III) Roche LightCycler® 480

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

IV) Roche LightCycler® Nano

Set the appropriate channel to SYBR® Green I detection.

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

V) BioRad DNA Engine Opticon® 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		

VI) 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 %	

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) 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	
	Gain-Optimisation			
	☐ Optimise gain before melt on all tubes			
	The gain giving the highest fluorescence less than will be selected			95

IX) 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		
		70	00:00:01	4	0.1	00:01
		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 59 to 62).

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* has to be performed with each sample testing (pages 47 to 50).

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 (Fig. 34, page 59, 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 54).

The specific peak can vary in height (part 2 of Fig. 34, page 59; pink melting curves). In some cases, eluates of the samples can show one or two unspecific peaks (part 1 of Fig. 34, page 59, 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 Fig. 34, page 59, 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 has to 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.

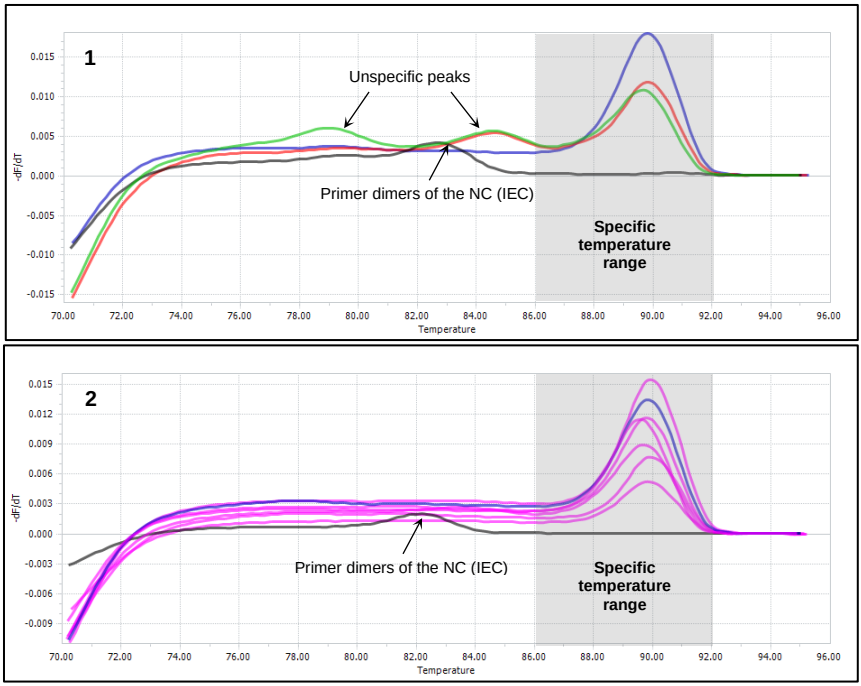
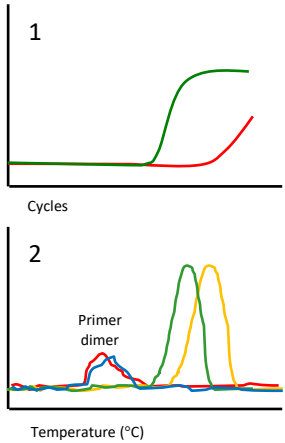


Fig. 34: Melting curve analysis (Roche LightCycler® 96) of a negative reference Internal Extraction Control (NC (IEC), black curve) and eluates of samples showing different peaks (coloured curves) in assay *MA Control*. The blue sample curve shows a specific peak (90°C) of the Internal Extraction Control. 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 60 to 62) 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 colour 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 this illustration it is understood that the positive PCR controls (P1 and P2) indicate full functioning of the assay.
Legend to pages 60 to 62:

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.

A

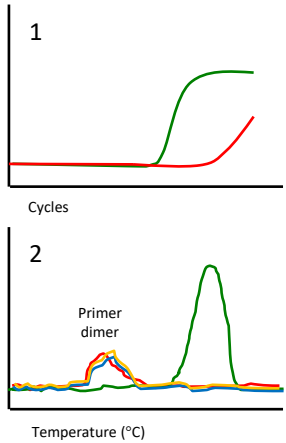


Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
+	-	+	-	+

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.

B

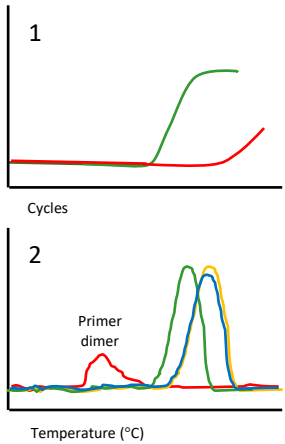


Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
+	-	-	-	+

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.

C

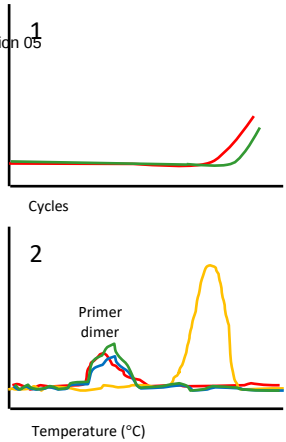


Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
+	-	+	+	+

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.

D



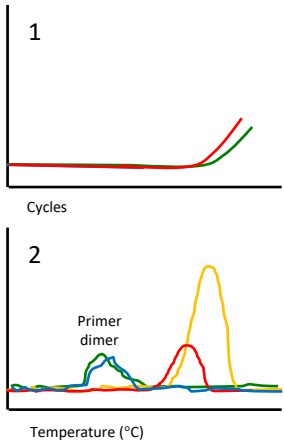
Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
-	-	+	-	-

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



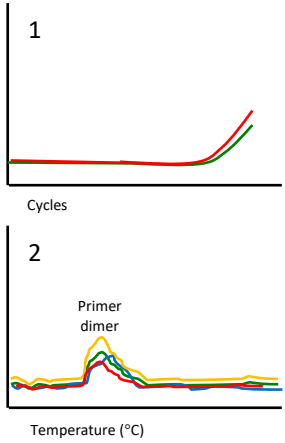
Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
-	+	+	-	-

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



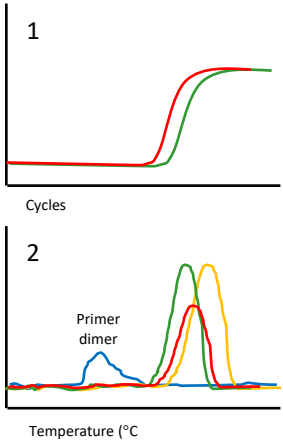
Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
-	-	-	-	?

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 has to be repeated.

G



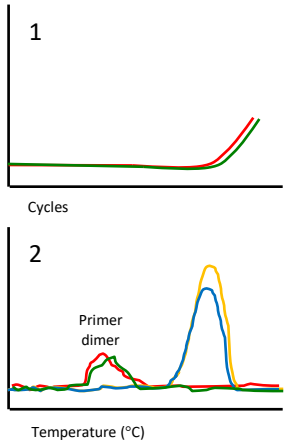
Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
+	+	+	-	?

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.

H



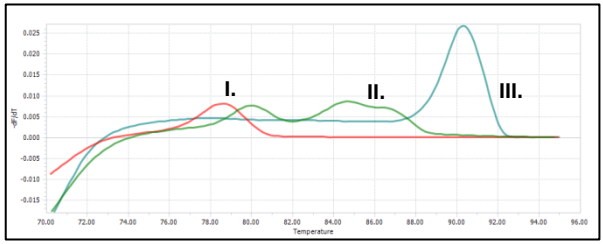
Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
-	-	+	+	?

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*) has to be repeated with more care to avoid contamination.

An example for a 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:
Phone: +49(0)421 69 61 62 0 • **E-Mail:** support@molzym.com

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	• Enzymes not used in the correct order.	Ensure that all enzymes are placed in the correct position in the reagents vial rack (coloured code).
	• Enzymes volume too low.	Ensure that all enzyme vials are briefly centrifuged before use. Make sure that the enzymes are not frozen if placed in the instrument
No pathogen DNA detectable in spiking tests with *SU* buffer	• Insufficient lysis	Ensure that all enzymes are placed in the correct position in the reagents vial rack (coloured code). Ensure that all enzyme vials are briefly centrifuged before use.
	• PCR inhibition	The *Extraction column* was clogged and inhibitors are co-eluted. The filter of the *Extraction cartridge* was moist/contaminated and the eluate volume higher than normal. Inhibitors like ethanol are co-eluted. The result of *MA Control* was negative. Repeat the extraction.
	• 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 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).
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. 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 the samples and buffers. Open buffer bottles 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 20). Clean the vacuum system (section 1F, page 42). Run cleaning program at least once a week (section 1G, pages 43).

	• Contaminated *Pipette tips*	Use **only** Molzym´s DNA-free Pipette tips (order no. D-925-0xy) to avoid DNA contamination. Do not use the remaining pipette tips in the used tip rows and do **not re-assemble** the tips in the pipetting tip racks to avoid contamination of following extractions
False negative PCR result (no signal in Assay *MA Control*, Internal Extraction Control)	• PCR inhibitors co-eluted	Ensure that all enzymes are placed in the correct position in the reagents vial rack (coloured code). Ensure that all enzyme vials are briefly centrifuged before use. Make sure that the eluate volume is in the range 70 to 140 µl. If higher, the column was clogged which leads to the co-elution of ethanol. Ethanol is a strong PCR inhibitor. In this case the extraction should be repeated. A volume <70 µl is possible and does not have an influence on the result.
No eluate	• Process error; elution buffer on the column.	Place the column into the *ET* tube, close the lid and briefly centrifuge the tube. **Caution:** Avoid contamination by handling. Wear sterile disposables (gloves, sleeve covers, lab coat), a disposable mask and protective goggles. The result of the sample analysis may deviate from the evaluated extraction process.
Eluate volume <70 µl	• The column was partial clogged with remaining particles of the sample (e.g., tissue).	Check carefully the Extraction Control Assay (MA Control) and if needed repeat the extraction.
Eluate volume >140 µl	• Malfunction of the drying of the column.	Check carefully the Extraction Control Assay (MA Control) and if needed repeat the extraction.
Error messages	• Error message indicated by an alarm sound when starting the extraction script.	The control board shows the following error code: 028-007-004-000 013-051-000-000 000-000-000-000 Switch off the instrument and start the extraction scrip once again. If the problem with the script persists, contact the technical support for help.
	• Error message during operation.	Switch off the instrument and start it again. Select the ‘Service Menu’ and choose ‘Display Error Log’. Note the error code. Contact the technical support for help.

Incorrect picking of Pipette tips in the instrument

- Rejected tips in the racks, no tip picking on positions

Depending on the number of samples, it is normal for pipette tips to remain in some positions in the racks. Check if this is the case, otherwise contact the technical support for help.

Remaining Pipette tip (orientation for position coordinates see Fig. 11, page 30)

Sample No.	**rows** (excluding full rows)		**positions**
1	1-4	rack 1	H, F, D, B
2	1-8	rack 1	H, F, D, B
3	1-8	rack 1	G
4	only full rows		
5	9	rack 1	G
6	12	rack 1	G, E
7	2-12 1-3 4	rack 1 rack 2 rack 2	G G H, G, F, D, B,
8	4	rack 2	G, E, C, A
9	4 6	rack 2 rack 2	G G, E, C, A
10	8	rack 2	H, G, E, F, D, B
11	2,4,5, 8,11 1,2,4, 5,7,8, 10.11	rack 1 rack 2	G G
12	only full rows		

Rejected sample positions

- The column was clogged and the position switched off during the extraction process

Cause: Incomplete solubilisation of the sample.

Fluid samples:

- Use only freshly collected samples or samples stored at +4 to +12°C for no longer than 2 days. For longer storage of fluid samples use Molzym´s *UMD-Tubes* (order no. Z-801-020) which contain a cryoprotectant.
- Sputum and cell culture are inappropriate for the SelectNA™*plus*. These fluid samples may clog pipette tips and the column in the instrument.
- Use for mucous fluids, purulent fluids and fluids with flakes of tissue or solid particles an enzymatic pre-treatment step. Ensure that samples do not contain particles after digestion (section 1B, pages 24 to 25). These samples may clog pipette tips and the column in the instrument without the pre-treatment.

Tissue samples:

- Ensure that samples do not contain particles after digestion (section 1B, pages 24 to 25).

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Tradenames

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ABI 7500 Fast®	Life Technologies
BigDye®	Applied Biosystems
BioBall® MultiShot 550 KBE	bioMérieux
Biosphere®plus	Sarstedt
CFX96™	BioRad
DNA Engine Opticon®	BioRad
GuardOne® Werkbank	Starlab
LABWASH® Premium Classic	VWR Chemicals
LightCycler® 1.5, 2.0, 96, 480 and Nano	Roche
Mastercycler®	Eppendorf
Meliseptol® New Formula	B. Braun
Micro-Dx™	Molzym
Mx3000P® and Mx3005P®	Stratagene
PCR strip of 4, 200 µl, Biosphere® plus	Sarstedt
peqStar 96Q	peqlab
QiaQuick®	Qiagen
Reliant®	Lonza
RipSeq®	Pathogenomix
Rotor-Gene®	Qiagen
SelectNA™*plus*	Molzym
S-Monovette®	Sarstedt
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Technical Support

If you have questions please call us.

Our hotline: +49(0)421 69 61 62 0 • **E-Mail:** support@molzym.com

Material safety data sheets are available on request.

Also visit Molzym's homepages for further information:
http://www.molzym.com

Technical Service

The maintenances of the instrument should be done on a yearly basis. For further information consult the technical service.

Order Information

Product	Contents and Application	Cat. No.
***Micro-Dx*™** (CE IVD)	**24** reactions **48** reactions Automated pathogen DNA isolation and PCR detection.	U-200-024 U-200-048
SelectNA™*plus* necessary for processing of the *Micro-Dx*™.	**Instrument** for automated pathogen DNA extraction for 1 to 12 samples of whole blood, other body fluids, swabs and tissues.	D-400-001

Other Products supplementary to *Micro-Dx*™

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Pipette tips* SelectNA™*plus, DNA-free Necessary for processing of the *Micro-Dx*™.	**2x [2x 96]** tips **4x [2x 96]** tips **8x [2x 96]** tips	D-925-024 D-925-048 D-925-096
Waste bags* SelectNA™*plus Necessary for processing of the *Micro-Dx*™.	**500** bags	D-928-500
Cleaning Cartridges for the SelectNA™*plus* instrument.	**12** cartridges	D-927-012
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