Micro-Dx

REF **U-200-024 / U-200-048**

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

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

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** –

BRUKER

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Content Page

Kit Information

Kit Content – *Micro-Dx*

Automated DNA Isolation	Σ 24 (U-200-024)	Σ 48 (U-200-048)	**Single use**
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	No
TSB	1x 25 ml	2x 25 ml	No
PKB	1x 7.5 ml	2x 7.5 ml	No
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	Σ 24 **(U-200-024)**	Σ 48 **(U-200-048)**	**Single use**
Kit 3 - PCR Reagents (store at -15 to -25°C), in white boxes			
MA Bac, Mastermix assay bacteria, 2.5x conc.	2x 0.30 ml	2x [2x 0.30 ml]	No
MA Yeasts, Mastermix assay yeasts, 2.5x conc.	2x 0.30 ml	2x [2x 0.30 ml]	No
MA Control, Mastermix assay control, 2.5x conc.	1x 0.36 ml	2x [1x 0.36 ml]	No
MolTaq 16S/18S	3x 0.05 ml	2x [3x 0.05 ml]	No
H_2O - DNA-free PCR-grade water	3x 0.75 ml	2x [3x 0.75 ml]	No
DS - DNA staining solution, 10x conc.	2x 0.30 ml	2x [2x 0.30 ml]	No
Kit 4 - PCR Controls & Detection Reagents (store at -15 to -25°C)			
A) Detection Reagents (in white box)			
LS - Gel loading solution, 6x conc.	1x 0.4 ml	1x 0.4 ml	No
SM - DNA size marker	1x 0.24 ml	1x 0.24 ml	No
SeqGP16 - Sequencing primer (bacteria)	1x 0.1 ml	2x 0.1 ml	No
SeqGN16 - Sequencing primer (bacteria)	1x 0.1 ml	2x 0.1 ml	No
SeqYeast18 - Sequencing primer (fungi)	1x 0.1 ml	2x 0.1 ml	No
B) Positive PCR Control (in transparent bag)			
DNA Standard P1, for PCR positive control runs	1x 0.3 ml	1x 0.3 ml	No
DNA dilution buffer (for P1)	1x 25 ml	2x 25 ml	No
Consumables PCR Detection & Identification (store at +18 to +25°C), in Kit 1			
MT - Mastermix tubes, 1.5 ml (for Kit 3)	2x 50	3x 50	
Manuals (in Kit 1)	Σ 24 **(U-200-024)**	Σ 48 **(U-200-048)**	**Single use**
Manual	1x	1x	No
Short manual sheets	5x	5x	No

Symbols

Symbols used in labelling and in the manual, e.g. section 'Hazard and Precautionary Statements' (pages 12 to 13).

Informative symbols

LOT	Batch code	Σ	Contains sufficient for <n> tests		Keep away from sunlight
	Biohazard	Cont.	Content of the package		Manufacturer
REF	Catalogue number		Do not re-use		Temperature limit, store at
	Caution	CE	European conformity		Use-by date
	Consult instructions for use	IVD	In vitro diagnostic medical device		Warning; Sharp element
CH REP	Swiss authorised representative				

Hazard pictograms

	Flammable		Toxicity		Health hazard
	Corrosive		Irritant		Environmentally Hazardous

Storage and Stability

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

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

Guarantee for full performance of ***Micro-Dx*** as specified in this manual is only valid if storage conditions are followed Table 1 on page 6).

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

Kit 1 (Buffers & Consumables):

Buffers, cartridges and consumables must be stored at room temperature (+18 to +25°C).

Buffers, cartridges and consumables must be stored away from sunlight.

Kit 2 (Enzymes):

Take care that the vials of the DNA Isolation (Kit 2) unit must 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) must 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 away from sunlight.

Do not freeze again (*DS*, *SM* and *LS*) and store at +4 to +12°C for further use. After use, *MA Bac*, *MA Yeasts*, *MA Control* 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) must be stored at -15 to -25°C upon delivery and must be stored at +4 to +12°C after the first handling.

Table 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				
PKB				
ST tubes				
Plus-SV vials				
Buffer cartridges				
Extraction columns				
Extraction cartridges				
ET tubes				
Kit 2 - Enzymes & Reagents:				
Enzyme K, MolDNase C, BugLysis plus and *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	exp. date
H2O		+18 to + 25°C	+4 to + 12°C -15 to -25°C	1 (thereafter freeze) exp. date
DS (DNA Staining Solution)		+18 to + 25°C	+4 to + 12°C	exp. date
Kit 4A - Detection Reagents:				
LS (Gel loading solution)	-15 to -25°C	+18 to + 25°C	+4 to + 12°C	exp. date
SM (DNA size marker)			+4 to + 12°C	exp. date
SeqGP16, SeqGN16, SeqYeast18			-15 to -25°C	exp. date
Kit 4B - Positive PCR Control:				
P1 DNA Standard	-15 to -25°C	+18 to + 25°C	+4 to + 12°C	exp. date
P2 (prepare freshly for the PCR), 1:500 dilution of P1	none			1
DNA Dilution Buffer	-15 to -25°C			exp. date
Consumables PCR Detection & Identification, in Kit 1:				
MT - Mastermix tubes, 1.5 ml (for Kit 3)	+18 to + 25°C	+18 to + 25°C	+18 to + 25°C	exp. date

Intended Use and Indication

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 mainly primary-sterile specimens.

Micro-Dx is intended for body fluids, swabs and for tissues (validated specimens see Table 3 page 8). 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 Table 2.

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

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

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 non-primary sterile sample materials, or for frozen sample material stored without freeze protection. Do not use specimens other than those mentioned below.

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

Transport media including 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.

Inappropriate sample material:

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

These materials may clog the Pipette tips and Extraction columns which will cause the Pressure Monitoring System (pages 34 to 35) to reject the sample to prevent an overflow of the Extraction column and contamination of the instrument and other samples.

Do not use other specimens than validated in Table 3.

Table 3: ***Micro-Dx*** is validated with the following specimens.

Fluid samples	**Swabs**	**Tissue samples**
Ascites aspirates	Bones	Abscesses
BAL (bronchoalveolar lavage)	Mouth	Aorta
Blood (EDTA or citrate stabilized)	Nasopharynx	Artificial tissues
Blood cultures	Wounds	Biopsy sphenoid sinus
CSF (cerebrospinal fluid)		Bone marrow
Joint aspirates		Heart valve
Mucus		Lung tissues biopsy
Plasma		Pacemakers
Platelet concentrates		Paraffin blocks
Pleural fluid		Pericardium
Pericardial effusion		Prostheses (e.g., heart valve)
Synovial fluid		
Urine		

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 kit:

- **SelectNA*plus*** DNA extraction instrument (Molzym order no. D-400-001).
- Additional components required for the SelectNA*plus* instrument:
  - ***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., VWR, Darmstadt, Germany) or a bench top microcentrifuge (e.g., miniSpin, Eppendorf, Germany)
- Sample positive control (run control):
  e.g., BioBall® MultiShot 550 KBE, bioMérieux, Germany
  - BioBall® MultiShot *Candida albicans* NCPF 3179 (56003)
  - BioBall® MultiShot *Escherichia coli* NCTC 12923 (56006)
  - BioBall® MultiShot *Staphylococcus aureus* NCTC 10788 (56009)
- 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., 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 57 to 63)
  Optional: Real-Time PCR instruments are validated (pages 70 to 72)
- 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, QIAquick® PCR Purification Kit, Qiagen (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 (70.3010.255)
  - 100 µl type Eppendorf (70.3030.255)
  - 300 µl type Eppendorf (70.3040.255)
  - 1000 µl type Eppendorf (70.3050.255)
- 1.5 ml micro tubes, e.g., Biosphere® plus, Sarstedt, Germany (72.706.200)
  - For the preparation of the positive PCR control P2
  - For gel electrophoresis
- PCR tubes, e.g., PCR strip of 4, 200 µl, Biosphere® plus, Sarstedt, Germany (72.990)
- Surface decontamination, e.g., Meliseptol® New Formula (rapid disinfectant, ethanol containing), B. Braun, Germany 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.

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 49). 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 13):
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 13):
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 13):
H310-H317-H318-H361d-H411;
P273-P280-P301+P310-P302+P352+P310-P305+P351+P338

Lysis buffer, prefilled in *Buffer cartridges* (W0, Figure 23, page 43)

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

Warning

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

Control buffer, prefilled in *Buffer cartridges* (W5, Figure 23, page 43)

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

Warning

Hazard and precautionary statements*(page 13):
H302-H315-H319-H332;
P280-P301+312-P304+P340+P312-P305+P351+P338

Binding buffer, prefilled in *Buffer cartridges* (W6, Figure 23**,** page 43**)**
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, Figure 23**,** page 43**)**
Contains ethanol (>50 %): **Flammable liquids and irritating (eyes)**.

Danger

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

Emergency information (24-hours service)
For emergency medical information, please contact the regional poison centre in your country.

* **H225:** Highly flammable liquid and vapour; **H302:** Harmful if swallowed; **H310:** Fatal in contact with skin; **H312:** Harmful in contact with skin; **H314:** Causes severe skin burns and eye damage; **H315:** Causes skin irritation; **H317:** May cause an allergic skin reaction; **H318:** Causes serious eye damage; **H319:** Causes serious eye irritation; **H332:** Harmful if inhaled; **H334:** May cause allergy or asthma symptoms or breathing difficulties if inhaled; **H336:** May cause drowsiness or dizziness; **H361d:** Suspected of damaging the unborn child; **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 CE-marked as an **in vitro diagnostic medical device** in accordance with Directive 98/79/EC. Where placed on the market on or after 26 May 2022, **Micro-Dx** continues to be placed on the market in accordance with the applicable transitional provisions of Regulation (EU) 2017/746 (IVDR), meeting the conditions set out therein for devices compliant with Directive 98/79/EC.
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 detection of bacteria and fungi by 16S and 18S rRNA PCR.
Micro-Dx is used in combination with the **SelectNA*plus*** instrument which provides 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 detect pathogens up to days earlier than culture and in patients who are negative in 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 depletion of host 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 depletion 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 is designed for broad-range detection of bacterial and fungal DNA based on conserved 16S and 18S rDNA target regions. Detectability depends on sequence compatibility, organism load, sample type, sample handling, DNA extraction efficiency and absence of PCR inhibition. Pathogens classified as safety level S3 or S4 are excluded (see ‘Contraindication page 7), including both culturable and non-culturable strains.. Sequence analysis of amplicons using primers provided with this kit is a confirmation of PCR results and allows the identification of strains detected in samples by BLAST analysis.

Test Principle

In its concept, ***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 (Figure 2, page 17). 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 depletion 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 bacterial and fungal 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, and swabs. For tissue biopsies a short manual protocol precedes the automated extraction of microbial DNA. After loading of the sample(s) into the instrument, the following protocol proceeds fully automated.

The pre-analytic procedure is based on the following four steps A to D (Figure 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 microbial cells potentially present in the sample on the membrane.

B In a series of following steps, the immobilized microbial cells 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.

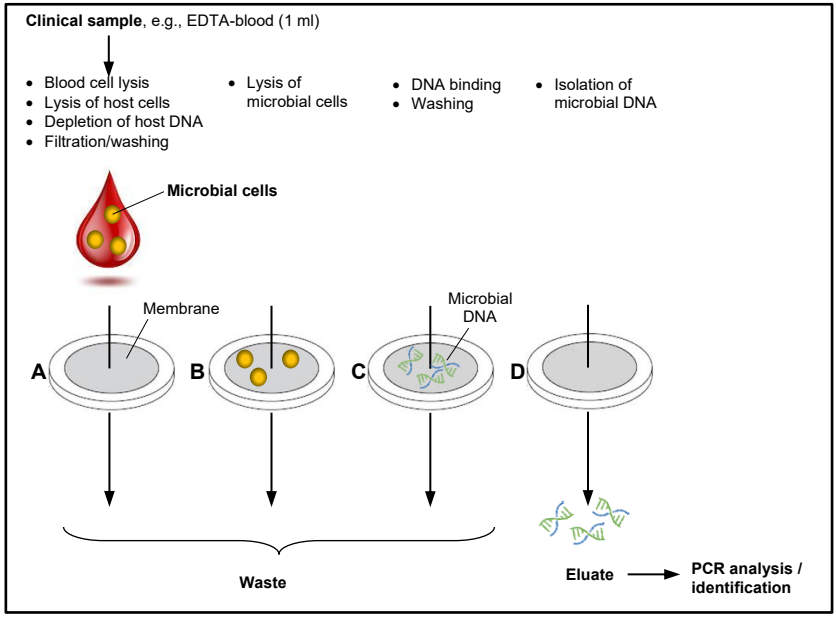


Figure 1: Scheme of the fully automated **Micro-Dx** procedure.

Part 2(a): PCR Analytics
DNA eluates (part 1, page 48) are used for broad-range 16S and 18S rDNA PCR analysis. Two assays are supplied, assay Bacteria (component *MA Bac*) and assay Yeasts (component *MA Yeasts*) enabling the sensitive detection of pathogens. 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 (component *MA Control*) is included in the kit.

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

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 (Figure 2; page 17). 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 Bacteria (component *MA Bac*) employs primers, *SeqGN16* and *SeqGP16*.
SeqGN16 targets mainly Gram-negative bacteria and including few gram-positive bacteria. The excluding few gram-negative species will be detected by *SeqGP16*.
SeqGP16 targets mainly Gram-positive bacteria and including few gram-negative species. The excluding species will be detected by *SeqGN16*. The primers are not strictly discriminative. For the exceptions of the sequencing primers consult table on https://www.molzym.com/images/services/Exceptions_of_Sequencing_Primers.pdf.

By using both sequencing primers in separate reactions, mixed strains of the two groups can be resolved. Sequencing of amplicons from the fungal assay (component *MA Yeasts*), is performed using *SeqYeast18*.
Bacterial taxa and fungal taxa (only *Candida* spp., *Cryptococcus* spp. and *Aspergillus* spp.) are identified with the help of the free online tool, SepsiTest-BLAST (https://www.sepsitest-blast.com). An overview of all species strains included SepsiTest-BLAST database is given in an Excel file which can be downloaded in the FAQ section of the SepsiTest-BLAST homepage (https://www.sepsitest-blast.com/en/faqs.php).

There are three potential outcomes of the sequencing analysis:

i) In case of readable sequences (Figure 2, A, readable), the BLAST online search may result 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: SepsiTest-BLAST is a quick tool for identification but does not contain all species. For comparison, we recommend to blast sequence results also with another tool e.g., NCBI BLAST https://www.ncbi.nlm.nih.gov/blast. The result with the best score is valid. For more information see section 2H) Analysis for Strain Identification on page 67.

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

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

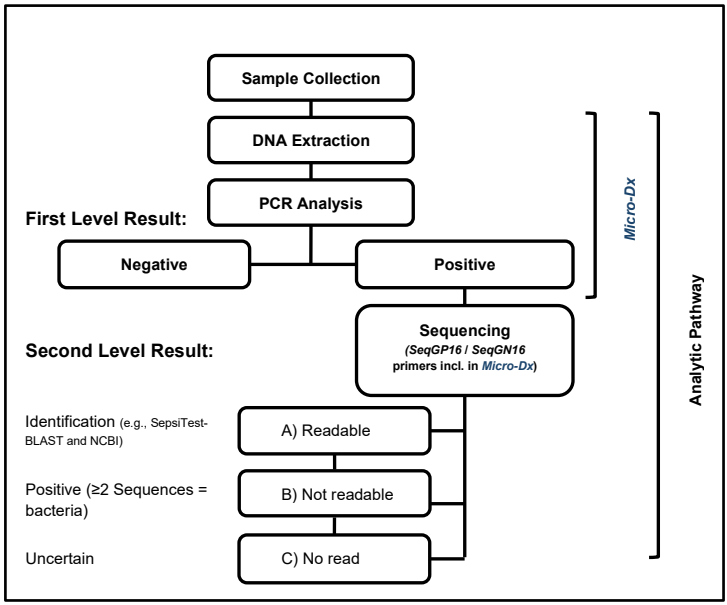


Figure 2: Detection of microorganisms in samples using ***Micro-Dx*** and sequencing analysis. The analytic pathway includes the detection and identification of bacteria and fungi (only bacteria shown).

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 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 (component *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)

Run Controls are not mandatory but recommended to run, e.g., once per reagent lot or in a determined time interval.

This control reflects the performance of the lysis and DNA extraction procedure from microorganisms and should be performed at least once per setup. There are two ways proposed to perform a run control:

i) Negative samples (buffer *SU*, Kit 1) are spiked with 100 to 1.000 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

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

Positive PCR Control

This test includes a definite number of target sequence copies to make sure that the assay is performing as specified. The *DNA Standard P1* (Kit 4B) comprises a mixture of DNAs extracted from *Bacillus subtilis* and *Saccharomyces cerevisiae*.

The set of controls comprises of a high (P1) and low (P2) standard DNA for Mastermix Assay Bacteria (*MA Bac*) and Mastermix Assay Yeasts (*MA Yeasts*). The high concentrated DNA standard (P1) is supplied with this kit and has a concentration of approx. 1.0 ng (*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 diluted 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 one mismatch (excluding terminal sites). As a result, more than 345 species are detectable, among which more than 200 species have been sequence-identified by *MolYsis* technology in clinical evaluations so far (Table 4).

Table 4: Extract of microorganisms identified in clinical evaluations.

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

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

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 14 to 17).

Analytical Sensitivity

Molzym's sample pre-treatment and DNA isolation constitutes the optimal solution for high sensitivity PCR and Real-Time PCR analysis of DNA from pathogenic bacteria and fungi.
By this combination, for instance, *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 Table 5.

Table 5: 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 (Table 6). 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 (Table 6). Among these, pathogens like *Candida parapsilosis* (heart valve), *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).

Table 6: Results of ***Micro-Dx*** evaluations using samples from routine diagnosis (see Table 3, page 8). 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

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 49).

- **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. Frequently change protective gloves during handling and change protective clothing when moving from one lab to another (below).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 34 to 35) 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; see below) 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

General Information

! We strongly recommend a user training before using the kit or SelectNA*plus* instrument for the first time.

! For the DNA extraction on the SelectNA*plus* instrument the room temperature is set to **+18 to +25°C**. 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 9 to 10.

! 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.

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.

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) Preparations for sample loading, page 28). Do not work under UV irradiation.

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 26 to 49). If this is not possible, store the samples in a refrigerator (+4 to +12°C). The stored samples should be analysed within two days after sample collection to avoid loss of microbial DNA.

! Use only fresh samples.
For blood collection, Molzym has evaluated K-EDTA and citrate S-Monovette® (Sarstedt, Germany) for the use with ***Micro-Dx***. The transport and storage of tissue specimen is recommended in a transportation liquid (e.g., physiological saline solution or *TSB* buffer of this kit).

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 a suitable cryoprotectant which stabilises fluid samples. Thaw samples to room temperature for extraction.

Do not use the following inappropriate sample material 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
- eNat® and eSwab® systems (Copan, USA)
- Samples with transport media including Agar, gel, charcoal medium and thioglycolate (e.g., Amies)

Procedure

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:

  - Pre-treatment buffers: SU (for fluid samples and swabs), TSB and PKB (for tissue)
  - *ST – Sample tubes* (for pre-treatment only; flip cap tubes, 2.0 ml)
  - *Plus-SV – Plus-sample vials* (for instrument; screw cap vials)
  - *Extraction columns*
  - *Extraction cartridges*
  - *Buffer cartridges*
  - *ET - Elution tubes* (flip cap tubes, 1.5 ml)

  The buffers and consumables should be kept away from sunlight, store it in the kit boxes.

  There are two different tubes for the sample preparation!

  - *ST* tubes (flip cap) are only for the pre-treatment steps for fluids (prepared with method 2), swabs and tissue (see section 1B) Preparations for sample loading, pages 28 to 31). Important, do not use the tubes in the instrument!
  - *Plus-SV* vial (screw cap) is only for usage in the instrument and for pre-treatment steps for fluids (prepared with method 1, see section 1B) Preparations for sample loading, page 28).

- **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 sample, use one 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 46).

  For the pre-treatment of tissue and some kind of fluid samples use *Enzyme K*. Place the *Enzyme K* in a cooling rack for usage and replace the *Enzyme K* vial to the freezer (-15 to -25°C) immediately after handling.

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

  Continue with section 1B) Preparations for sample loading, page 28.

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, pacemakers, prosthesis and other solid materials (see part *iii) Tissue samples*, page 30).

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 30).

Do not use other specimens than validated in Table 3, page 8.
Sample volume for the instrument is always 1 ml.

i) Fluid samples
(ascites, BAL (bronchoalveolar lavage), blood cultures, CSF (cerebrospinal fluid), 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.

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.

For each sample place a *Plus-SV* vial (screw cap, Kit 1) and an *ET* tube (*Elution tube*, Kit 1) in a rack, close the lid and mark the tubes with the sample ID.

Do not mark the *Plus-SV* vials on the lid, but rather the tubes on the white printed label.

1. Transfer of the fluid specimens:

   a) 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).

     Blood cultures (excluding cultures with activated carbon): Use up to 0.2 ml of the culture and fill up to 1 ml with buffer *SU*.

b) Method 2: Fluid samples with enzymatic pre-treatment

- For each sample place a *ST* tube (flip cap, Kit 1) in a rack, close the lid and mark the tubes with the sample ID.
- Pipette up to 0.8 ml fresh fluid sample from the sample container into *ST* tube (flip cap).

For difficult sample material, use less material (e.g., 0.3 to 0.5 ml) and fill up to 0.8 ml with buffer *SU*.

- 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.

2. Check that 1 ml sample volume is contained in the *Plus-SV* vial, fill up to 1 ml (use the measure line of the tube) with buffer *SU* (Kit 1), if required.
3. Then mix the sample by pipetting.
4. Transport the rack with the closed *Plus-SV* vials and *ET* tubes to the instrument. Continue with section ‘Loading Procedure of Components‘; page 35. For further details of the instrument see section 1C) Instrument Setup, pages 32 to 35.

Do not use the *ST* tubes (flip cap) in the instrument. Only for enzymatic pre-treatment of fluid samples (method 2).

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

Use only swabs without agar gel.

Swabs on agar gel media, eNat® and eSwab® systems (Copan, USA) 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.

For each sample place a *Plus-SV* vial (screw cap, Kit 1) and an *ET* tube (*Elution tube*, Kit 1) in a rack, close the lid and mark the tubes with the sample ID.

Do not mark the *Plus-SV* vials on the lid, but rather the tubes on the white printed label.

1. For each sample place a *ST* tube (flip cap) in a rack, close the lid and mark the tubes with the sample ID.
2. Pipette 1 ml of buffer *SU* (Kit 1) into a *ST* tube (flip cap tubes). If there is fluid in the swab vial, pipette 1 ml thereof into a *ST* tube instead of buffer *SU*.

   Remove the swab from the swab vial and transfer to the *ST* tube.
3. Wash the swab by swirling in the fluid and pressing to the wall of the *ST* tube several times. Thereafter, discard the swab.
4. Transfer the sample from the *ST* tube into a *Plus-SV* vial (screw cap vial).
5. Check that 1 ml sample volume is contained in the *Plus-SV* vial, fill up to 1 ml (use the measure line of the tube) with buffer *SU* (Kit 1), if required.
6. Then mix the sample by pipetting.
7. Transport the rack with the closed *Plus-SV* vials and *ET* tubes to the instrument. Continue with section 'Loading Procedure of Components'; page 35. For further details of the instrument see section 1C) Instrument Setup, pages 32 to 35.

Do not use the *ST* tubes in the instrument. Only for pre-treatment.

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

Tissue specimens are sampled under aseptic conditions and transported to the laboratory.

For each sample place a *Plus-SV* vial (screw cap, Kit 1) and an *ET* tube (*Elution tube*, Kit 1) in a rack, close the lid and mark the tubes with the sample ID.

Do not mark the *Plus-SV* vials on the lid, but rather the tubes on the white printed label.

1. For each sample place a *ST* tube (flip cap) in a rack, close the lid and mark the tubes with the sample ID.
2. Pipette 180 µl of buffer *PKB* (Kit 1) into a *ST* tube (flip cap tubes).

3. Transfer the specimen to a sterile support, e.g., a Petri dish, by using sterile forceps.
4. 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.
5. Transfer the dissected specimen to the *ST tube* filled with buffer *PKB.* The specimen should be covered completely by the buffer.
6. Add 20 µl of *Enzyme K* (Kit 2D) to the specimen. Pipette the enzyme into the buffer.
7. 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.
8. After incubation, transfer the fluid phase into a *Plus-SV* vial (screw cap vials) by pipetting. For this use the 200 µl pipette.

   Avoid transferring any particles that may clog pipette tips and the columns in the instrument.

   Optional, in cases the sample transfer in the *Plus-SV* vial is difficult:
   - Fill up to 1 ml with buffer *TSB* in the *ST* tube (flip cap tube).
   - Vortex the *ST* tube at full speed for 15 s and short centrifuge for 5 s (up to 2,000 x*g*).
   - Pipette fluid phase into a *Plus-SV* vial (screw cap vials) by pipetting. For this use the 200 µl pipette and **avoid transferring any particles**.
   - Continue with step 9.
9. Check that 1 ml sample volume is contained in the *Plus-SV* vial, fill up to 1 ml with the transport solution, if available, or with buffer *TSB* (use the measure line of the tube).

   Avoid transferring any tissue particles from the transport solution!
10. Mix the sample by pipetting.
11. Transport the rack with the closed *Plus-SV* vials and *ET* tubes to the instrument. Continue with section ‘Loading Procedure of Components’; page 35. For further details of the instrument see section 1C) Instrument Setup, pages 32 to 35.

    Do not use the *ST* tubes in the instrument. Only for enzymatic pre-treatment of tissue samples.

1C) Instrument Setup

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

The Instrument

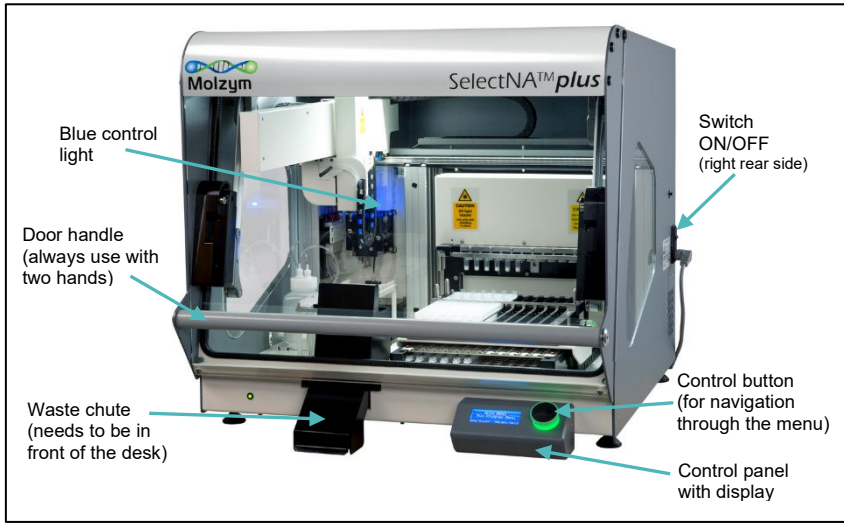


Figure 3: The outer features of the **SelectNA*plus*** instrument.

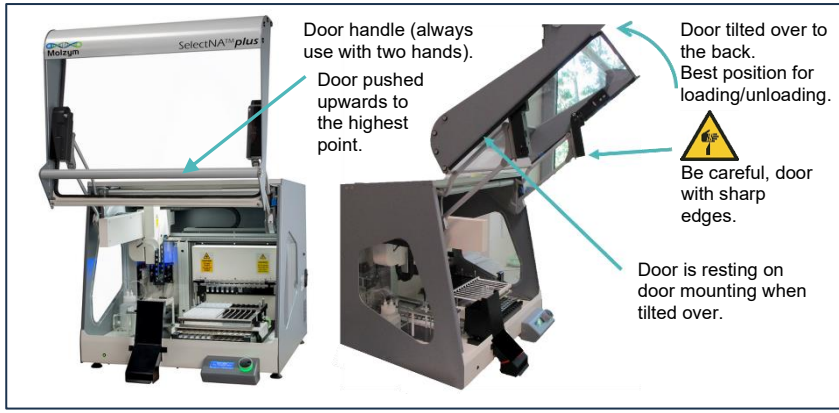


Figure 4: Handling of the door of **SelectNA*plus*** instrument. Open the door with both hands by pulling it slightly to yourself and then pushing it upwards. When it is at the highest point, tilt the door backwards until the top of the door is resting on the top of the door mounting.

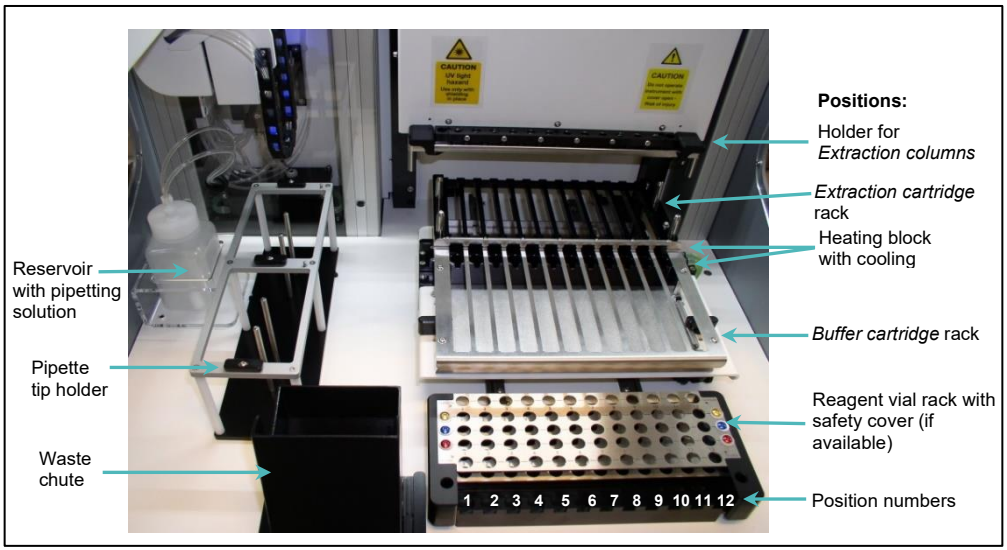


Figure 5: The interior of the **SelectNA*plus*** instrument.

Instrument Control Panel

Initialisation and selection of the programs of the instrument.

Figure 6: Control panel with Control button of the instrument.

- Turn ON the instrument on the right rear side of the instrument (Figure 3, page 32). The blue control lamp inside the instrument is on (Figure 3, page 32).
- Press the 'Control button' (Figure 6) of the front control panel to initialise the instrument.
- Select the section menu 'Run Program Menu' (page 35), 'UV decontamination' (page 49) or 'Cleaning Menu' (page 52) from the 'Main Menu' by turning the 'Control button' and press the button to select it.

Pressure Monitoring System

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

The system controls whether residual liquid is on 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. For all other positions, where no residual liquid is detected, the extraction process continues as usual. The switched off positions will be indicated at the end of the extraction program in the display as “rejected channel” (details on page 35).

The pipetting arm is equipped with a sensor box including the pressure monitoring system (Figure 7). The ***SelectNAplus*** 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 (Figure 7).

Figure 7: Pipetting arm with pressure monitoring system.

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 (Figure 8, part A). The column on sample position 1 is rejected. At the next block (samples 5 to 8) channel 3 is switched on (Figure 8, part C) and the sample position 7 is rejected, etc..

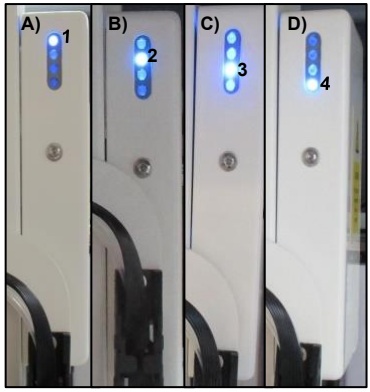


Figure 8: Four channels of the pressure monitoring system. During the extraction run, the blue LED is switched on if the corresponding sample position of the pipetting block is blocked.

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 (Figure 9, part A) and after confirming with the `Control button` with position 12 (Figure 9, part B).

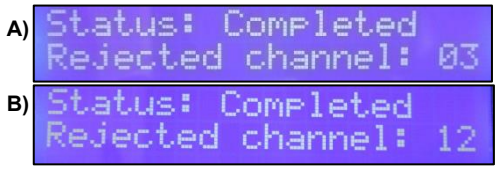


Figure 9: Display shows the rejected positions at the end of the program. Example: A) The first rejected position 3 and B) the second rejected position 12.

Loading Procedure of Components

Use Kits 1 (Cartridges & Consumables) and 2 (Enzymes). Preparation of the components for the following steps see part 1A) How to start on page 27.

Ensure that the instrument is UV-decontaminated before each extraction run (main menu: "UV decontamination", see 1D) Decontamination after each run step 12, page 51).

For each sample, use one 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 (step 8a), page 46).

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 37 to 49).

Load the instrument according to the following steps 1 to 10 (pages 36 to 48):

Turn the instrument on, press the 'Control button' to initialise the instrument (Figure 6, page 33). Press the 'Control button' to select no. 1 'SelectNAplus' and press the button.

1. Waste bag

Place a *Waste bag* (not supplied, order no. D-928-500) to the waste chute and fix with the rubber ring (Figure 10).

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.

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

Figure 10: Waste chute with fixed *Waste bag*.

Load the following components (steps 2 and 4 to 8) direct in the instrument.

2. Reservoir with pipetting solution

Open the door of the instrument (Figure 4, page 32). Check that the reservoir is sufficiently filled with pipetting solution (250 ml autoclaved deionized water; Figure 11, part 1, and Figure 5, page 33). The bottle holder edge can be used for the minimum fill level (blue arrow in Figure 11, part 1).

If needed, disconnect the reservoir with the pipetting solution from the tubing by slightly turning the connectors at the lid to open them (blue arrow in Figure 11, part 3). The tubes can now be pulled straight upwards to handle the reservoir bottle easier. To reconnect, push the connectors down and turn them slightly “click-system” (Figure 11, part 3).

If the instrument is not equipped with a clip for the tubing (Figure 11, part 2), make sure that the tubings are arranged on the left side of the reservoir (Figure 11, part 1), because otherwise the tubes may block the pipette tip pickup.

Confirm the loading step ‘Load DI water bottle (250 ml DI water)’ by pressing the ‘Control button’.

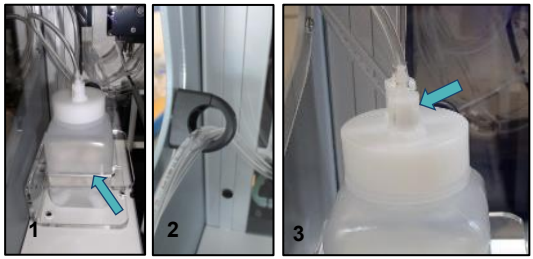


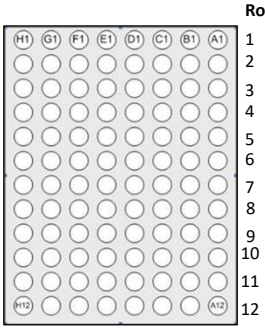
Figure 11: The reservoir with pipetting solution (part 1). Clip for fixing the tubing (part 2). Reservoir connected the tubing with two connectors (blue arrow, part 3).

3. 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' by pressing the `Control button`. Select `No` to correct the number of samples. By selecting `No` you come back to the `Main Menu`.

4. 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 Figure 12). 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. Table 7 shows the consumption of pipette tip rows for the number of samples, respectively. An example on page 39 is presented to explain the algorithm.

Figure 12: Pipette tip rack

Table 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:

Place the pipette tip holder on the top of empty Buffer cartridge rack (Figure 13).

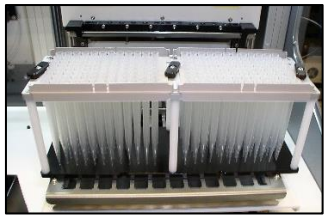


Figure 13: Loading position for the Pipette tip holder.

Carefully load the pipette tip holder (Figure 14, part 1) with pipette tip racks in the correct direction. Avoid letting the pipette tips come into touch with the tip holder. Fix the racks with the three black clips of the holder (Figure 14, part 2 and 3).

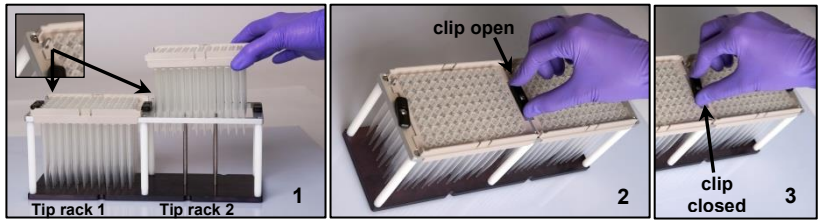


Figure 14: Loading procedure of pipette tip racks into the tip holder.

Place the loaded pipette tip holder into the instrument (Figure 15). Fit the notches of the holder in the four black knobs at the bottom of the instrument (Figure 15, part 2 and 3). Ensure that the pipette tip holder is pressed down and does not wiggle.

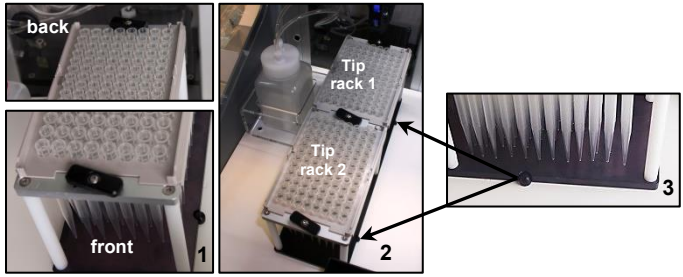


Figure 15: Loading *Pipette tips* into the instrument.

Be sure that there are enough filled tip rows to run the selected number of samples (Table 7, page 37). The display shows the required number of rows. Select the tip row position (A1 to A12) and the tip rack (1 or 2) in which the instrument shall start picking up tips. 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'.

Please note that all required full tip rows must be directly behind each other. It is not possible to start in tip rack 2 and continue in tip rack 1!

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 Figure 16) and run with 7 samples which consumed 16 tip rows (Table 7, page 37). Accordingly, the tip rack 1 is empty and the tip rack 2 has 8 left over rows completely filled with tips (Figure 16; tip rack 2 position row A5 to A12).

Reference to Table 7 (page 37) shows 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.

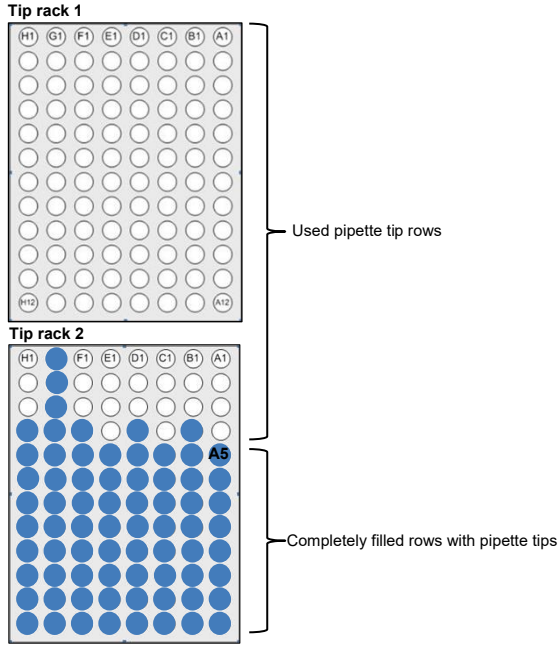


Figure 16: Pipette tip racks at the end of running 7 samples. White positions are empty, blue positions filled with pipette tips.

Remove the empty tip rack 1 and place the partially filled tip rack 2 (Figure 16, page 39) 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 (Table 7, page 37). Continue loading the instrument with the other consumables and reagents and the sample as below.
For processing 11 to 12 samples use two new full tip racks.

Confirm `Yes` for the selected `starting tip full row` by pressing the button and continue with step 5 'Extraction columns' (page 40).
Select 'No' to return to step 3 `Selection of the number of samples` (page 37).

5. *Extraction columns*

For loading the *Extraction columns*, place the column rack on the empty Buffer cartridge rack (Figure 17).

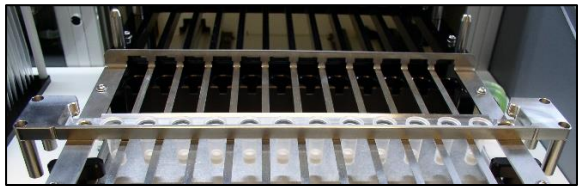


Figure 17: Loading position for the column rack on the empty Buffer cartridge rack.

Note the orientation of the column rack. The front is marked with the position numbers (if available).

Pick the columns with a sterile forceps and place them into the column rack from the left to the right side (Figure 18).

Note that on both ends of the column rack are two smaller holes into which the *Extraction columns* cannot be placed (Figure 18, part 2, marked with blue arrow, right smaller hole not visible in picture).

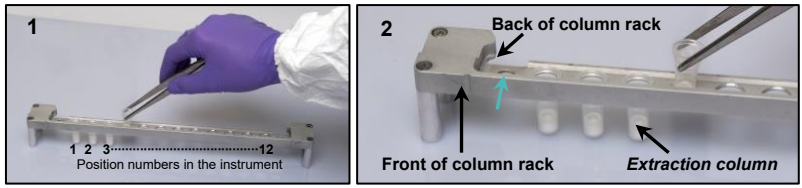


Figure 18: Loading procedure of *Extraction columns* in the column rack.

Open the clips of the black holder of the column rack (Figure 19, part 1). Place the filled rack into the instrument in the holder. Push the column rack completely into the holder (Figure 19, part 1).

Note: Moving the holder in and out might require some force. Press against the open clips with your thumbs to achieve a leverage effect.

Fix the rack with the black clips on each side of the holder (Figure 19, parts 2 and 3).

Confirm this loading step ‘Load Extraction columns’ by pressing the ‘Control button’.

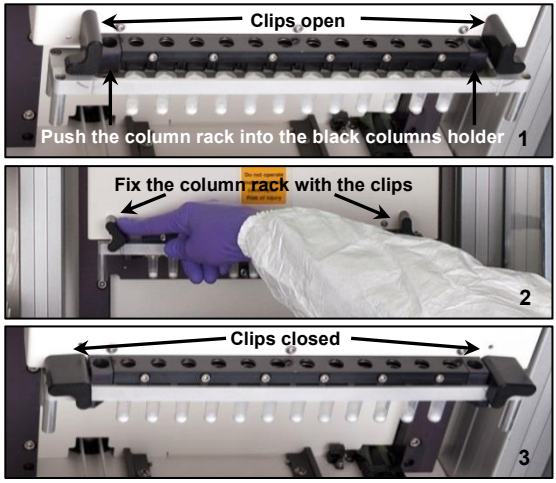


Figure 19: Loading procedure of the filled column rack into the instrument.

6. *Extraction cartridges*

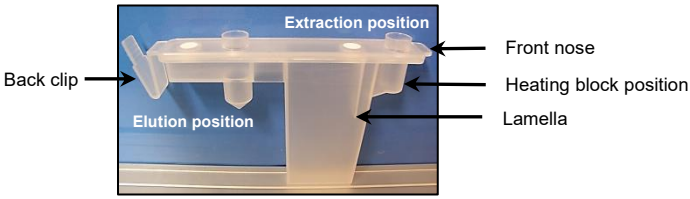


Figure 20: *Extraction cartridge*

For loading the *Extraction cartridges* (Figure 20), open the black clips on both sides of the Extraction cartridge rack, remove it and place it on the top of the empty Buffer cartridge rack (Figure 21, part 1). Load the extraction rack with the *Extraction cartridges* (Figure 20), starting from the left to the right side (position numbers 1 to 12, Figure 21, part 2).
Place the front nose of the *Extraction cartridge* in a slanted angle under the metal edge (Figure 21, part 2).
Push the *Extraction cartridge* into the rack until the back clip locks in position with a click sound (Figure 21, part 3).

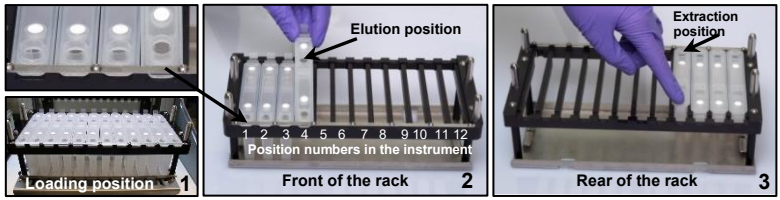


Figure 21: Loading procedure of *Extraction cartridges* into the extraction rack.

Place the rack into the instrument. Take care that cartridges are placed with the extraction position in the corresponding position in the heating block. Fix the rack with the black clips on both sides (Figure 22).
Confirm this loading step 'Load Extraction cartridges' by pressing the 'Control button'.

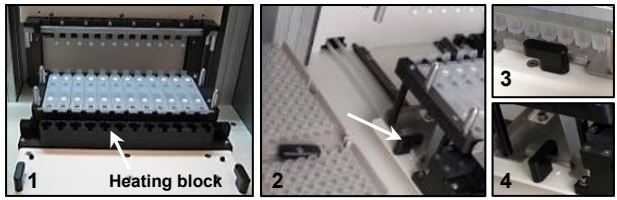


Figure 22: Loading procedure of the filled Extraction cartridge rack into the instrument. Heating block for Extraction cartridges and Buffer cartridges (part 1). The rack is fixed on both sides by black clips (left clip, white arrow part 2). Clip open (part 3) and clip closed (part 4).

Do not load the *Extraction cartridges* without moving the Extraction cartridge rack to the loading position (Figure 21, part 1). Cartridges might not be inserted properly and the heating block can be damaged

7. *Buffer cartridges*

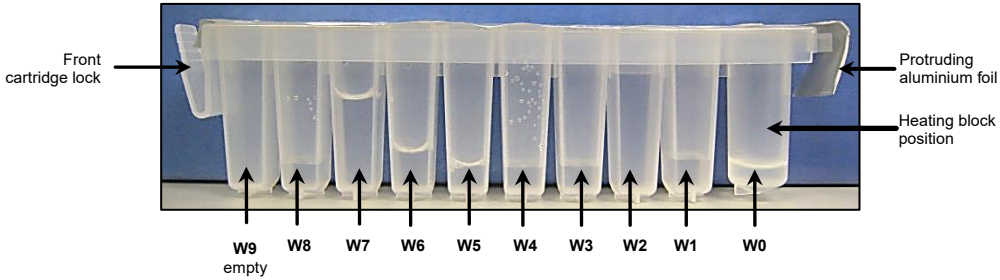


Figure 23: Filled *Buffer cartridge* (DNA-free).

For loading the *Buffer cartridges*, open the black clips on both sides of the Buffer cartridge rack, lift it up and place it in front of the heating block.

Bend the protruding aluminium foil at the rear of the *Buffer cartridge* upwards and load the buffer rack with the *Buffer cartridges* (Figure 23). Place the front nose of the *Buffer cartridge* in a slanted angle under the metal edge (Figure 24, part 1). Push the *Buffer cartridge* in the rack until the back clip locks in position with a click sound (Figure 24, part 2).

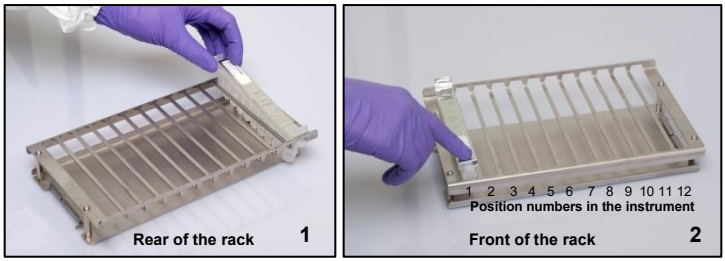


Figure 24: Loading procedure of *Buffer cartridges* into the rack (constructed representation, figure show only the placing of the *Buffer cartridges* in the rack).

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 (Figure 25). Fix the rack with the black clips on both sides (Figure 25).

Figure 25: Loading procedure of the filled buffer rack into the instrument. The rack is fixed on both sides by black clips (black arrows, part 1). Clip open (part 2) and clip closed (part 3).

Carefully peel off the aluminium foil by pulling constantly and slightly directed to the side (Figure 26, 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' by pressing the 'Control button'.

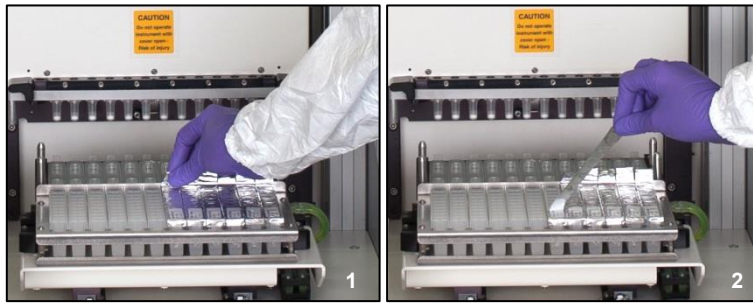


Figure 26: Removing of the aluminium foil of the *Buffer cartridges*.

Do not load the *Buffer cartridges* without moving the Buffer cartridge rack out of the heating block. Cartridges might not be inserted properly and the heating block can be damaged.

8. 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.

Keep your hands off the instrument!

After movement of the rack with *Extraction* and *Buffer cartridges*, open the door (Figure 4, page 32). Place the Reagent vial rack into the instrument, if not already in.

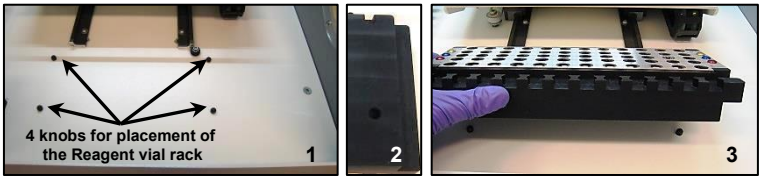


Figure 27: Placing Reagent vial rack in the instrument.

For this, place the notches on the bottom of the rack (Figure 27, part 2) into the four black knobs at the bottom of the instrument (Figure 27, part 1). For this, slide the rear bottom notches of the rack in the rear knobs (Figure 27, part 3) and then place the front bottom notches in the corresponding knobs. Confirm the loading step ‘Open door & Load reagent vial rack` by pressing the ‘Control button’.

Place the following vials in the sequential steps (parts 8a) to 8c), page 46) in the Reagent vial rack of the instrument (Figure 28, page 46). Place the vials from left to right (without lids).

Color code: Each row of enzyme vials is marked with a colored knob at the left and right side of the Reagent vial rack, the colors on the Reagent vial rack match the colors of the enzyme vial lids.

The row for the *Plus-SV vials* is marked with ‘Blood’ or ‘Sample’ and the row for the ET tubes is marked with ‘Eluate’.

The screw caps of the enzyme and *Plus-SV* vials (step 8a) to 8c), page 46 to 47) can be disposed to the waste bag.

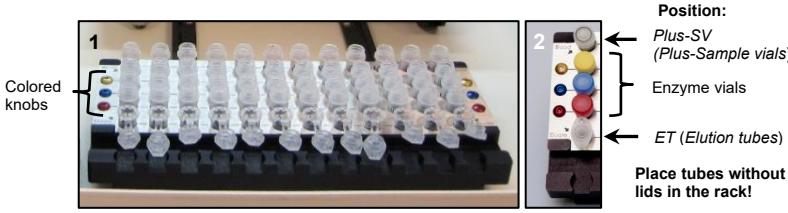


Figure 28: Reagent vial rack loaded with the components Plus-SV vials, enzyme vials with color code (colored knobs on the rack) and ET tubes (part 1).

Part 2 (constructed representation, components are only placed in the rack without lids) shows the positions of the tubes in the reagent vial rack including the color code of the enzymes.

8a) Enzymes and color code

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

! Remove the rack with enzyme vials from the freezer. Make sure that the enzyme vials have been pulse centrifuged to clear the lids (section 1A) How to start, page 27).

First, open the *BugLysis plus* vials (yellow cap) and place them into the Reagent vial rack at the position in row with the yellow colored knob, following positions 1 to 12 in the Reagent vial rack from left to right (Figure 28). Dispose the screw caps to the waste bag. Confirm the step 'Load Enzymes (yellow cap)' by pressing the 'Control button'.

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 colored knob, following positions 1 to 12 in the Reagent vial rack from left to right (Figure 28). Dispose the screw caps to the waste bag. Confirm the loading step 'Load reagents rack, Enzymes blue cap' by pressing the '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 colored knob, following positions 1 to 12 in the Reagent vial rack from left to right (Figure 28). Dispose the screw caps to the waste bag. Confirm the step 'Load Enzymes (red cap)' by pressing the 'Control button'.

8b) *Elution tubes* (*ET* tubes)

Open the lids of the *Elution tubes*, marked with the sample ID (section 1B) Preparations for sample loading, pages 28 to 31). Place the opened *Elution tubes* to the elution position following positions 1 to12 of the Reagent vial rack from left to right (Figure 28). Adjust the lids of the tubes to the front of the rack (part 1; Figure 28, page 46). Confirm the loading step 'Load Elution tubes' by pressing the 'Control button'.

8c) *Plus-Sample vials* (*Plus-SV*)

Remove the screw cap from each *Plus-SV* (see part B, page 28) containing the sample and place into the Reagent vial rack following positions 1 to 12 from left to right (Figure 28, page 46). Dispose the screw caps to the waste bag. Confirm the loading step 'Load Plus-Sample vials' by pressing the 'Control button'.

8d) *Safety cover (if available)*

After loading of the Reagent vial rack with enzymes, *Elution tubes* and the *Plus-SV* place the Safety cover onto the rack, if available for the instrument (Figure 29).
No information on the display for this step.

Figure 29: Safety cover for the Reagent vial rack.

9. Check the loaded instrument

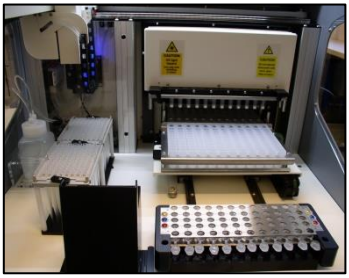


Figure 30: Loaded instrument.

Check that the aluminium foils of the *Buffer cartridges* have been removed (Figure 29).

Check, that all vials stand correctly at the same level in the rack, all caps have been removed and Safety cover is placed on the Reagent vial rack, if available (Figure 29). Confirm this step `Check all caps removed` by pressing the 'Control button'.

Check that a waste bag is fixed at the waste chute and the exit of the chute/the bag is open and tips can fall into the bag. Otherwise, pipette tips may pile up in the chute and fall into the interior of the instrument. Further information see step 1, page 36.

10. Start the extraction process

Close the door of the instrument and confirm this step 'Close door and press button to start' by pressing the 'Control button'. The instrument now starts the extraction.

Do not open the instrument during the extraction run.

The program is finished with a signal sound. For the approx. total time of the extraction program for the corresponding number of samples see Table 8.

Table 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

11. Eluted DNA – end of the extraction process

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 34 to 35) will be shown in the display, when applicable. Every rejected position needs to be confirmed by pressing the 'Control button'.

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 an 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 analysis reaction. In this case, the extraction should be repeated.

Decontamination of the Instrument

You need low-lint, soft paper towels for the cleaning procedure. For disinfection employ Meliseptol® New Formula (B. Braun, Germany) or an ethanol containing disinfectant or alternative ready-to-use moist disinfectant wipes (low-lint and with the recommended disinfectant).

After use, dispose the paper towels or wipes in the waste for infectious material.

Do not spray surfaces inside the instrument with the disinfectant. Instead use paper towels soaked 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).

   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 let it incubate for 10 min.
3. Remove the Reagent vial rack from the instrument. Wipe the surface of the instrument around the rack with a paper towel soaked with disinfectant or ready-to-use disinfectant wipes.
4. The display of the Control panel indicates: '**WARNING** Transferring rack. Press button'. Close the door.

   Press the button and the rack with the Extraction cartridges and Buffer cartridges transfers forward.

   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 or in container for infectious material. 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.

   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.
6. Store 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.

Take care to avoid handling-borne contamination and follow the advice for avoidance of contamination on page 22.
Do not use the remaining pipette tips in the uncomplete rows and do not re-assemble the tips in the pipetting tip racks to avoid contamination of following extractions.

7. Clean the removed racks (Pipette tip holder, racks for Extraction cartridges and Buffer cartridges, Reagent vial rack) and the Safety cover (if available). For this, wipe the racks with a paper towel soaked with disinfectant or ready-to-use disinfectant wipes and wait for 1 min or the specified time of exposure of the disinfectant. Let the racks dry on air.

8. Clean the suction cups and the surfaces of the instrument with a paper towel soaked with disinfectant or ready-to-use disinfectant wipes. Let the surfaces dry.

Do not spray the interior of the instrument!
Do not clean the pipetting arm, the control panel, the chains, cooling tubes and the windows of the instrument with disinfectant.

9. Replace all cleaned racks to the instrument. Place the column rack to the right side of the pipette tip holder for UV cleaning (Figure 31, part 1).
Place the cleaned safety cover of the reagent vial rack (if available) on the right side of the instrument. The underside of the cover faces the inside of the instrument (Figure 31, part 1 and 2). The cover is placed on the black feet (Figure 31, part 2).

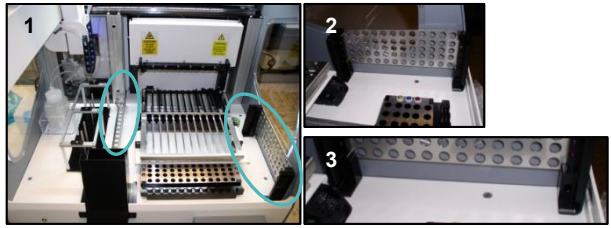


Figure 31: Interior of the instrument for UV decontamination of the column rack (Blue marking on the left side of the picture, part 1) and safety cover (blue marking on the right side of the picture, part 1 and part 2 + 3).

10. Check that minimum 100 ml pipetting solution (autoclaved deionized water) is still in the reservoir. The bottle holder can be used for the minimum fill level (blue arrow in Figure 11, page 36). If not, fill the reservoir completely (max. 250 ml) with autoclaved deionised water (Figure 11, page 36). Remove the decontaminated waste chute (after 10 min time of exposure, see step 2). Wipe the inside of the waste chute with a paper towel soaked with disinfectant or a ready-to-use disinfectant wipe.

Important, change the contaminated gloves after wiping the waste chute. Dispose the gloves in the waste bag or container for infectious material.

11. Thereafter place the cleaned waste chute back into the instrument. Press the ‘Control button’ to finalise the protocol.
12. Close the door. Wipe the door handle and the door top with the disinfectant and select the ‘UV decontamination’ from the ‘Main Menu’ by turning the ‘Control button’. Confirm the program by pressing 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. Confirm this with the ‘Control button’.

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

13. Start a new extraction run or switch off the instrument.

Do not re-use material (*Buffer* and *Extraction cartridges*, *Extraction columns*, *Plus-SV* vials, enzyme vials and *ET* tubes) from the SelectNA*plus*.

Discard Waste bag including used components form the SelectNA*plus* instrument as infectious waste according to your institution’s procedures.

Note: If the waste chute has impurities that cannot be removed by decontamination after the extraction run, the waste chute can also be cleaned as follows.

Optional cleaning procedure of the waste chute:

1. Remove the decontaminated waste chute (see above) from the instrument.
2. Clean the chute in a laboratory dishwasher using a mildly alkaline cleaning powder with sodium hydroxide (e.g., LABWASH® Premium Classic, VWR).

   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.
3. Dry the cleaned waste chute with a low-lint, soft paper towel.
4. Thereafter place the waste chute back to the instrument.
5. Close the door and select ‘UV decontamination’ from the ‘Main Menu’ by turning the ‘Control button’. Confirm the step ‘Start decontamination’ by pressing the ‘Control button’. The instrument now starts the UV decontamination. The program is finished with a signal sound. Confirm this with the ‘Control button’ and switch off the instrument.

The instrument must be emptied before starting the UV decontamination program. The racks, column holder, safety cover and pipette tip holder as well as unused pipette tips and the pipetting solution may remain inside the instrument.

1E) Cleaning script - Cleaning of the pipetting system

Clean the pipetting system every 14 days.

1. Supplied material to be used: 4 *Cleaning cartridges* (Figure 32; supplied with the instrument, Molzym order no. D-927-012) and *Cleaning bottle* (Figure 32).

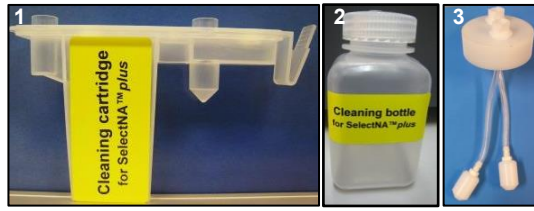


Figure 32: *Cleaning cartridge* (1), *Cleaning bottle* (2) and lid (3).

2. 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. The bottle holder edge can be used for the minimum fill level (blue arrow in Figure 11, page 36).
3. Select the cleaning script 'Cleaning Menu' from the 'Main Menu' for cleaning the pipetting tubes by turning the 'Control button', confirm by pressing the button.
4. Load the four *Cleaning cartridges* (Figure 32) 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 Figure 21 and Figure 22, page 42). Confirm the step by pressing the 'Control button'.
5. Remove the reservoir bottle with the pipetting solution incl. the lid. The tubing is fixed with a click-system on the lid. Turn slightly to open the connectors (Figure 11, part 3, page 36) and the tubing can be removed.
6. Remove the lid from the reservoir and place it on the *Cleaning bottle*. Connect the filled *Cleaning bottle* with the pipetting tubes in the instrument. Attach the connectors of the tubing to the lid and turn slightly to fasten 'click'. Confirm the step 'Load cleaning bottle (100 ml 1 % bleach)' by pressing the 'Control button'.
7. 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.
8. In the meantime, 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.
9. After the 'soaking (wash)' step (10 min, indicated with a signal sound), open the door and remove the *Cleaning bottle* incl. the lid.
10. Clean the tubing of the lid (outside) with a low-lint, soft paper towel soaked with the 1 % bleach solution and rinse with autoclaved deionized water.
11. Fill the reservoir bottle with 250 ml autoclaved deionized water, remove the lid from the *Cleaning bottle* and close the reservoir bottle with it.

12. Connect the cleaned reservoir bottle (filled with autoclaved water) with the tubing of the instrument. Close the door of the instrument and resume the program by pressing the ‘Control button’ to rinse the pipetting tubes.
13. In the meantime, empty the *Cleaning bottle* and rinse with autoclaved deionized water. Let the bottle dry on air before closing it with the screw cap.
14. The program is finished with a signal sound. Open the door and confirm this by pressing the ‘Control button’.
15. Remove the cartridge rack from the instrument.

Do not take out the *Cleaning cartridges* from the rack while fixed in the instrument. This may damage the racks and heating block.

16. Dispose the solution from the *Cleaning cartridges* and rinse with autoclaved water. Dry the *Cleaning cartridges* on air for re-use in the next cleaning procedure.
14. Clean the cartridge rack with a paper towel soaked with disinfectant or ready-to-use disinfectant wipes and wait for 1 min or the specified time of exposure of the disinfectant. Let the rack dry on air.
17. Replace the empty and dry rack into the instrument.
18. Close the door of the instrument, select the ‘UV decontamination’ from the ‘Main Menu’ by turning the ‘Control button’ 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. Confirm by pressing the ‘Control button’ and switch off the instrument.

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 % in the *Cleaning cartridges*).

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

1F) Cleaning of the vacuum system

Cleaning of suction cups every 14 days.

Clean the white suction cups of the vacuum system, the black holder of the column rack (Figure 33) and the column rack by wiping. Do not spray the interior of the instrument!

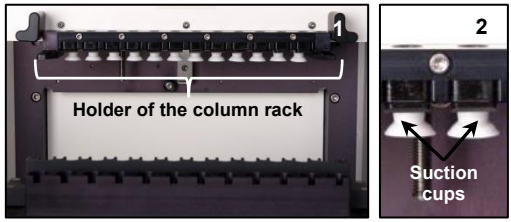


Figure 33: Holder of the column rack (1) and suction cups (2) of the vacuum system.

For this, pull the suction cups from the system and clean with a paper towel soaked with disinfectant (Figure 34) or ready-to-use disinfectant wipes and wait for 1 min or the specified time of exposure of the disinfectant. Let the suction cups dry on air. Push the dry suction cups back to their position in the column holder.

Figure 34: Cleaning of the suction cups.

For further information see ‘Troubleshooting’ point ‘No pathogen DNA detectable in spiking tests with SU buffer’ on page 81.

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

Important notes before starting

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

Note: The term 'mastermix' refers to the mix of the components of Table 9, page 62. Assay Bacteria, Assay Yeasts and Assay Control refers to the assay of the analysis. The component names (*MA Bac*, *MA Yeasts* and *MA Control*) are also used as synonyms for the assays.

Description of the Assays

With this unit, PCR assays are supplied for the testing of the presence of bacterial and fungal DNA in clinical specimens. The PCR assay Bacteria (*MA Bac*, Kit 3) and assay Yeasts (*MA Yeasts*, Kit 3) are based on primers that bind to conserved regions of the 16S (V3/V4 region) and 18S (V8/V9 region) rRNA genes of bacteria and fungi, respectively.
The tests comprise a two-step procedure including the use of **i)** the assays (components *MA Bac* and *MA Yeasts*; Kit 3) for the PCR amplification of sequences using extracted DNA (pages 24 to 48) 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 69 to 77).
The components *MA Bac* and *MA Yeasts* are 2.5x concentrated solutions, the final volume of the reaction mixture being 25 µl. This PCR Detection unit contains all components necessary for PCR runs.
The PCR assay Control (component *MA Control*, Kit 3) is a control for the performance of the extraction process and to confirm the absence of co-eluted PCR inhibitors together with the sample DNA. The assay tests the presence of the *Control DNA* (Internal Extraction Control Unit), which is added in the extraction process and is included in the eluate of each sample. The correct function of the extraction process and the absence of PCR inhibitors potentially co-eluted with the target pathogen DNA are monitored by PCR assay Control.

Protocols for amplification are supplied for the following instruments:

- Thermal Cycler (protocol, page 63):
  - Mastercycler® Eppendorf
- Real-Time PCR instruments (addendum pages 70 to 72):
  - LightCycler® 96, 480 and Nano, Roche
  - DNA Engine Opticon®, CFX96™, BioRad
  - Mx3000P®, Mx3005P®, Stratagene
  - ABI 7500 Fast®, Life Technologies
  - Rotor-Gene®, Qiagen
  - peqStar 96Q, peqlab

If using other instruments, make sure that the Assays Bacteria, Yeasts and Control (*MA Bac*, *MA Yeasts* and *MA Control)* perform correctly with the cycler. For *MA Bac* and *MA Yeasts*, perform PCR reactions using PCR DNA Standard P1 and P2 which both should result in an amplification product. For *MA Control* a negative sample control (*SU* buffer, Kit 1) is extracted and tested in the assay. See PCR Detection, section 2A to 2E for the procedure (pages 60 to 66).

PCR Assays

Assays Bacteria (*MA Bac*) and Yeasts (*MA Yeasts*):

With each assay an extra volume is supplied to run PCR controls. Negative PCR controls should always be performed, at least with 10 % of the sample runs, to test for potential cross-contamination or other carryovers resulting from handling or air-borne errors. Also, with each set of sample runs positive PCR controls (page 18) must be included to control the performance of the assay. Follow the instructions for the performance of positive PCR controls (pages 60 to 63).

Assay Control (*MA Control*):

The Assay Control (*MA Control*) is an Internal Extraction Control test to check the performance of the extraction process and to confirm the absence of co-eluted PCR inhibitors together with the sample DNA. The assay must be performed with each sample testing (pages 60 to 63).

Validity of results:

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

Exceptional cases:

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

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

Packaging, Storage and Handling

Store all vials in this unit (Kits 3 and 4) at -15 to -25°C upon receipt.
For more information about the storage and stability of the PCR reagents see Table 1 (page 6) and in section 2A) How to Start (page 60).

The purification and packaging of the assays supplied in this PCR detection unit are performed under standard precautions for the avoidance of air-borne and handling-based DNA contaminations.
Take care to maintain a DNA-free environment during opening the vials and handling the components by working under a UV-decontaminated workstation. Use only certified microbial DNA-free pipette tips and PCR consumables recommended for running the assays (pages 9 to 10).

MA Bac, MA Yeasts and MA Control (Kit 3)

The assays are supplied as a 2.5x concentrated solution in DNA-free screw cap vials (Kit 3). For usage, the assays are thawed at room temperature (+18 to +25°C). After use, the assays can be stored in the refrigerator (+4 to +12°C) for further use at the same day, but should be replaced to -15 to -25°C for longer storage.

MolTaq 16S/18S (Kit 3)

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

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

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

<u>*DS* (including SYBR® Green1, Kit 3)</u>

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

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

<u>H_2O</u>

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

<u>*SM* (including SYBR® Green1, Kit 4A)</u>

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

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

<u>*LS* (Kit 4A)</u>

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

<u>*DNA dilution buffer* and *DNA Standard P1* (Kit 4B)</u>

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

Quality Control and Specifications

Negative PCR controls using supplied DNA-free water instead of eluate are used for routine detection of contamination by microbial DNA in the purified final mastermixes (*MA Bac* and *MA Yeasts*). Guarantee is given for the absence of signals in negative controls at a rate of ≤5 %, provided the avoidance of contamination by handling errors.

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

PCR Detection

2A) How to Start

! For equipment, consumables and reagents to be supplied by the user see pages 9 to 10.

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

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

! For each pipetting use fresh tips.

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

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

Places where Handlings are performed

Symbols and explanation of the PCR working places:

DNA-free

Work under a PCR UV workstation. Use components of **Kit 3** and **consumables** (*MT*, Mastermix tubes for Kit 3) in **Kit 1**.
For the preparation of assays Bacteria, Yeasts and Control (components *MA Bac, MA Yeasts* and *MA Control*).

DNA handling

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

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

PCR Assays

Per assay, the following PCR reactions must be run:

Assay Bacteria (*MA Bac*)

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

Assay Yeasts (*MA Yeasts*)

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

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

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

DNA-free

Before starting with the preparation of the mastermixes (section 2B, page 62):

DNA-free

Kit 3:

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

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

DNA

Kit 4B:

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

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

     Tubes used must be DNA- and DNase-free (e.g., see page 9).
  3. Add 2 µl *P1* into the tube (with DNA dilution buffer) and vortex to mix.
  4. Briefly centrifuge to clear the lid.
  5. Label the dilution with '*P2*' and the preparation date.

Storage of the PCR Reagents after Handling

For more information about the storage and stability of the PCR reagents see section Packaging, Storage and Handling (page 58) and Table 1 (page 6).

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

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

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

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

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

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

Positive PCR control *P2* (needed for step 8, page 62)
Diluted DNA solutions (P2) tend to be unstable. **Do not use** the *P2* for **longer than 24 hours** and store it at **+4°C to +12°C**.

2B) Setup of the Assays

Keep all PCR tubes filled with mastermix chilled in the cooling racks, until placing in the PCR cycler. Cooling of the PCR tubes and the *MolTaq 16S/18S* is important to minimize the generation of primer dimers.

DNA-free

Preparation of mastermixes

1. **Arrange the required PCR tubes (strips or plates); not supplied) for all mastermixes (*MA Bac*, *MA Yeasts* and *MA Control*) in a PCR cooling rack (-15 to -25°C).**
2. **Briefly centrifuge *MolTaq 16S/18S* (Kit 3) and place it in the cooling rack (-15 to -25°C).**
3. **Use a *MT* (*Mastermix tube* 1.5 ml for Kit 3; in Kit 1) for *MA Bac*, another *MT* tube for *MA Yeasts* and a third *MT* tube for *MA Control*.**
   Place the *MT* tubes in a cooling rack. Pipette the supplied components of Kit 3 into each *MT* tube as shown in Table 9. Vortex the tubes to mix and briefly centrifuge.
4. **Pipette 20 µl of the mastermix *MA Bac* into each PCR well dedicated for samples, P1, P2 and NC, respectively. Repeat the procedure with mastermix *MA Yeasts*. Pipette 20 µl of the PCR-ready mastermix *MA Control* into each PCR well dedicated for samples and NC, respectively.**
5. **Add 5 µl *H_2O* (DNA-free water; Kit 3) into the NC PCR wells. Close all PCR wells with caps.**

DNA handling

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

Sample loading (template loading)

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

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

8. **Vortex the *P1* and prepared dilution *P2* (998 µl *DNA dilution buffer* and 2 µl P1, see 61) vials and pulse centrifuge. Pipette 5 µl of positive PCR control *P2* into a PCR well containing *MA Bac* and *MA Yeasts*, respectively. Repeat with *P1*. Close PCR wells with caps.**
9. **Continue with section 2C PCR Thermocycling page 63.**

Table 9: Preparation of mastermixes (Kit 3). Component volumes in µl.

reactions	*MA Bac, MA Yeasts or MA Control*	*H_2O*	*DS*	*MolTaq 16S/18S*
1	10.0	7.5	2.5	0.8
2	20.0	15.0	5.0	1.6
3	30.0	22.5	7.5	2.4
4	40.0	30.0	10.0	3.2
5	50.0	37.5	12.5	4.0
6	60.0	45.0	15.0	4.8
7	70.0	52.5	17.5	5.6
8	80.0	60.0	20.0	6.4
9	90.0	67.5	22.5	7.2
10	100.0	75.0	25.0	8.0

2C) PCR Thermocycling

Transport all chilled PCR tubes (strips or plates; -15 to -25°C) prepared as above to a place where PCR runs are performed. Program the Mastercycler® (Eppendorf) as described (Table 10). After the PCR run go to section 2D for the detection of amplicons.

Table 10: PCR program for Mastercycler® (Eppendorf)

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

2D) Detection by Agarose Gel Electrophoresis

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

! For equipment, consumables and reagents to be supplied by the user (pages 9 to 10).

! The DNA staining solution (*DS,* including SYBR® Green1), which is present in the mastermixes during PCR amplification, is used for gel electrophoretic visualisation of the amplicon DNA.

Make sure that the tubes are kept in the dark until gel electrophoresis.

Before starting with the preparation of gel electrophoresis:

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

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

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

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

  - *LS* (Kit 4A)

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

For more information about the storage and stability of the PCR reagents see section Packaging, Storage and Handling (page 58) and Table 1 (page 6).

Protocol:

Prepare a gel (2 %) following the instructions of the manufacturer or prepare a 2 % (w/v) agarose gel in 1x TAE buffer. Place the gel in a tray, transfer into the chamber and fill with freshly prepared 1x TAE running buffer as instructed by the manufacturer (the gel should be covered with approx. 1cm buffer).

1. Per PCR reaction, pipette 8 µl of the PCR product into a sterile 1.5 ml polypropylene tube (not supplied, DNA- and DNase-free) or in a well of a 96 well plate (not supplied, DNA- and DNase-free) and mix with 2 µl of the gel loading solution (*LS*). Mix by pipetting in and out for several times.
2. Pipette the mixture (10 µl) into an indentation of the gel. Repeat the procedure with the other PCR products, including samples, Internal Extraction Controls, positive PCR controls (P1 and P2), negative PCR controls.
3. Pipette 5 µl of supplied DNA size marker (*SM*).

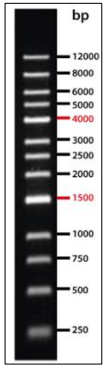


Figure 35: DNA size marker (*SM*, 1kb ladder) as a reference for gel electrophoretic detection of bacterial (*MA Bac*), fungal (*MA Yeasts*), and internal extraction control (*MA Control*) amplicons.

4. Close the electrophoresis chamber with the cover and run the gel at 10V/cm interelectrode distance in the dark.
5. 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.
6. Remove the gel, place under a UV lamp or on a transilluminator (260 to 310 nm wavelength) and photograph / document. Compare appearing bands of samples with the DNA size marker and positive PCR controls P1 and P2. For an example, see Figure 36 and Figure 37, pages 65 to 66.
   Note: If all bands show a too weak fluorescence signal, the gel can be re-colored in e.g., an ethidium bromide staining bath, if necessary.
7. Make sure that bands appear within the samples in the Assay Control (*MA Control*; Internal Extraction Control). This is important in cases of negative samples. Bands in this assay indicate the absence of PCR inhibitors in the eluates and the correct performance of the extraction process.

2E) Guidance to the Interpretation of PCR Results

A typical image of the results of the analysis of samples is given in Figure 36 for the Assay Bacteria (*MA Bac*) and Figure 37 (page 66) for the Assay Yeasts (*MA Yeasts*), including Assay Control (*MA Control*, Internal Extraction Control) in each figure (right side).

In this case, samples were collected from four patients and processed as described in the previous sections.
The controls are as expected, i.e. positive PCR controls P1 and P2 show bands at the correct position (approx. 450 bp for bacterial DNA in Figure 36 and approx. 310 bp for fungal DNA in Figure 37).
The negative PCR controls (NC) do not show a signal, so DNA contamination can be excluded.
The Internal Extraction Controls of the samples B1 through B3 (Figure 36) and samples Y1, Y3 and Y4 (Figure 37, page 66) show clearly visible bands (approx. 200 bp), demonstrating the function of the extraction process. The results from the Internal Extraction Controls, positive PCR controls and negative PCR controls indicate that the assay performed correctly.

Two of the samples show bands (samples B1 and B2) in the assay *MA Bac* at the expected gel position (Figure 36, left side) and thus are positive for bacterial DNA. Samples B3 (Figure 36) is negative with a positive signal in the assay *MA Control* (C3). This is a valid negative result for sample B3. Sample B4 was negative too, but no signal in assay *MA Control* (C4). This indicates inhibition in the sample and the result is invalid.

Exceptional cases:
In the case of specific bacterial or fungal bands in the samples tested (e.g., sample Y2, Figure 37) but lacking signal in the Internal Extraction Control (C2, Figure 37, page 66), the positive fungal result for the sample is valid.

All positive PCR samples must be identified by sequencing.

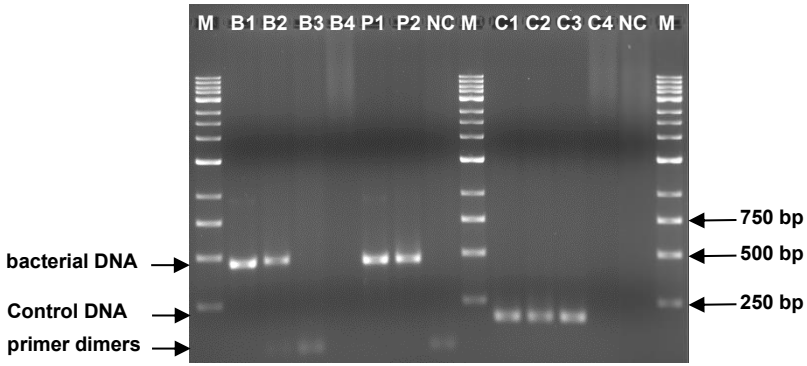


Figure 36: PCR analysis of eluates from four patient samples (B1 to B4) using Assay Bacteria (*MA Bac*). *P1, P2*: Positive PCR controls; NC: Negative PCR controls; C1 through C4: Internal Extraction Controls (*MA Control*) with respective eluates from patient samples B1 to B4 (banding at approx. 200 bp; C4: negative); M: DNA size marker (*SM*).
Signals at the position 'bacterial DNA' (450 bp) indicate that samples of patients B1 and B4 contain bacterial DNA (sequencing result: *Staphylococcus aureus*). The weak signals in sample B3 and NC at approximately 50 bp (arrow 'primer dimers') are the results of the unspecific binding in negative amplicons.

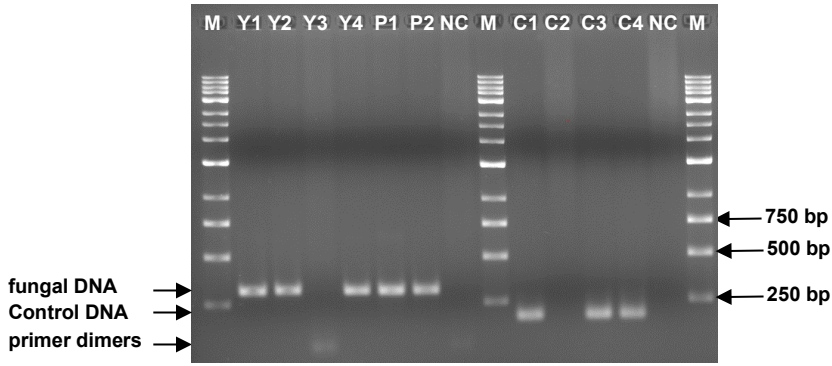


Figure 37: PCR analysis of eluates from four patient samples (Y1 to Y4) using Assay Yeasts (*MA Yeasts*, fungi). P1, P2: Positive PCR controls; NC: Negative PCR control; C1 through C4: Internal Extraction Controls (*MA Control*) with respective eluates from patient samples Y1 to Y4 (banding at approx. 200 bp; C2: negative); M: DNA size marker (SM). Signals at the position 'fungal DNA' (310 bp) indicate that samples of patients Y1, Y2 and Y4 contain fungal DNA (sequencing result: *Candida albicans*). The weak signals in sample Y3 at approximately 50 bp (arrow 'primer dimers') are the results of the unspecific binding in negative amplificants.

Identification of Pathogens by Sequencing of Amplicons

All positive PCR samples can be identified by sequencing.
Sequencing of amplicons together with BLAST online homology search is used for the identification of pathogens detected by ***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. (https://www.sepsitest-blast.com). For further investigations see section 2H, page 67.

2F) Purification of Amplicons

For sequencing, amplicons need to be purified. Qiagen's QIAquick® PCR Purification Kit (cat. no. 28104) is recommended 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 69 to 77) and follow the instructions of the manufacturer of the kit. Elute the purified amplicon from the column (QIAquick®) with **30 µl** sterile deionised water. Continue with the sequencing procedure (section 2G Sequencing, page 67).

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).

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

For sequencing, use the sequencing primers (10 µM each) supplied with Kit 4. For sequencing of amplicons from Assay *MA Bac* (bacteria) use *SeqGP16* and *SeqGN16* in separate reactions, and from Assay *MA Yeasts* (fungi) use *SeqYeast18*. *SeqGP16* and *SeqGN16* are primers binding to regions within the amplicon specific for Gram-positive and Gram-negative bacteria, respectively. *SeqGN16* targets mainly Gram-negative bacteria, including few gram-positive bacteria. The excluding gram-negative species will be detected by *SeqGP16*. *SeqGP16* targets mainly Gram-positive bacteria, including few gram-negative species. The excluding species will be detected by *SeqGN16*.
The exceptions of the sequencing primers are available at the following link: https://www.molzym.com/images/services/Exceptions_of_Sequencing_Primers.pdf

As an example, the following protocol for QIAquick®-purified amplicons using the ABI Prism310® may give satisfying results. Use 2 µl of purified DNA for cycle sequencing. Add 4 µl Big-Dye® Reaction mix (containing polymerase und nucleotides), 0.5 µl sequencing primer *SeqGP16*, *SeqGN16* or *SeqYeast18* (10 pmol/µl) and PCR-grade water to fill up to a final volume of 20 µl. Incubate in a PCR machine under the following conditions: Initial denaturation at 95°C for 1 min; 26 cycles at 95°C for 30 s, 55°C for 30 s and 60°C for 4 min. To remove dye terminator molecules from sequencing samples, use your internal validated process. Combine 5 µl of the eluate containing the products of the sequencing reaction with 20 µl formamide (or TSR reagent containing formamide) and incubate at 95°C for 4 min. Apply the reaction mix to the capillary of the ABI Prism310®.
Validate the performance of the used sequencing system. For this, analyse the purified amplicons of the positive PCR controls P1 and P2. Both controls should give readable results. Alternatively use an overnight sequencing service (e.g., Eurofins Genomics, Germany).

2H) Analysis for Strain Identification

Molzym has developed SepsiTest-BLAST, a free online service (https://.sepsitest-blast.com), for the identification of bacteria and fungi based on small subunit rRNA genes. The identification relies on an algorithm for the comparison of input sequences with a reference sequence data library. SepsiTest-BLAST is characterized by a pool of more than 7.000 quality-controlled complete sequences of the 16S and 18S rRNA genes of only cultured and denominated eubacteria, *Candida* spp., *Cryptococcus* spp. and *Aspergillus* spp. The tool is very simple to use, and results are obtained as an output of hits in the order of decreasing sequence identity scores.
An overview of all species covered by the SepsiTest-BLAST database is given in an Excel file which can be downloaded in the FAQ section of the SepsiTest-BLAST homepage (https://sepsitest-blast.com/en/faqs.php).
Please note: SepsiTest-BLAST is a quick tool for identification but does not contain all species. For comparison, we recommend to blast sequence results also with another tool e.g., NCBI BLAST https://www.ncbi.nlm.nih.gov/blast. The result with the best score is valid.

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

the coverage of the sequence should also be high (>99%) and the length of the aligned segment should be >350 bp for bacterial and >200 bp for fungal sequences.
Sequence identities below 97 % are rejected by SepsiTest-BLAST. This may be the result of reading errors of the sequencing reaction. In such a case it is recommended to inspect the densitogram read-out for overlying sequences indicating the presence of more than one strain in the sample (Figure 2, page 17). Overlying bacterial sequences can be resolved using a specialised tool, RipSeq® (Pathogenomix; https://www.ripseq.com).

Addendum: Real-Time PCR Protocols

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

Please note that ***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® 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 72 to 77) 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 (starting page 56). 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 60 to 63).

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

The PCR run conditions are as according to the protocol described on pages 60 to 63. The specific thermocycling conditions are described on pages 70 to 72. For the preparation of mastermixes, follow the instructions (part 2, sections 2A to 2C, pages 60 to 63). 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 72).

! For equipment, consumables and reagents to be supplied by the user see pages 9 to 10. In addition, the following items are needed to perform Real-Time PCR:

- 1x Real-Time PCR machine (above). Other instruments must be validated for their use.
- PCR tubes (strips or plates), e.g., PCR strips (8x 0.2 ml) for various systems; e.g., Biozym Flat Optical 8-Cap Strip (order no. 712100)

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

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

Real-Time PCR Thermocycling and Detection by Melting Curve Analysis

A melting curve analysis has always to be performed in order to discriminate possible primer dimer formation from true bacterial or fungal signals. See examples on pages 72 to 77. All positive Real-Time PCR sample results have to be identified by sequencing. For sequencing of amplicons see part 2, section 2F) Purification of Amplicons to 2H) Analysis for Strain Identification, pages 66 to 67.

I) Roche LightCycler® 96

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

II) Roche LightCycler® 480

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

III) Roche LightCycler® Nano

Set the appropriate channel to SYBR® Green I detection.

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

IV) BioRad DNA Engine Opticon® and CFX96™

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

V) ABI 7500 Fast®

Switch off the ROX reference.

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

VI) Qiagen Rotor-Gene®

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

Amplification				
Method	**Cycles**	**Target Temperature [°C]**	**Incubation time [hh:mm:ss]**	**Acquisition Mode**
Hold	1	95	00:01:00	
Cycling	40	95	00:00:05	
		55	00:00:15	
		72	00:00:30	Acquiring to cycle A; Acquiring channel A

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

VII) Stratagene Mx3000P® and Mx3005P®

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

VIII) Peqlab peqStar 96Q

Method	Cycles	Target Temperature [°C]	Incubation time [hh:mm:ss]	Ramp rate [°C/s]	Step	Step Holding Sec.
Hold Stage	1	95	00:01:00	4		
PCR Stage	40	95	00:00:05	4		
		55	00:00:10	4		
		72 (Sampling)	00:00:25	4		
Melting Stage	1	95	00:00:01	4		
		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 74 to 77).

Validity of results:

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

Exceptional cases:

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

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

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

The Assay *MA Control* (Internal Extraction Control; Kit 3) is a test to check the performance of the DNA extraction process and to confirm the absence of co-eluted PCR inhibitors together with the sample DNA. *MA Control* must be performed with each sample testing (pages 60 to 63).

For the interpretation of the Assay results use only the melting curve analysis and ignore the Ct values (amplification curve).

The temperature of specific and potentially unspecific peaks depends on the used Real-Time PCR instruments. In the following sections examples are presented using the Roche LightCycler®96. Here, the specific peak of the Internal Extraction Control is located at approximately 90°C (Figure 38, page 73, 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 69).

The specific peak can vary in height (part 2 of Figure 38, page 73; pink melting curves). In some cases, eluates of the samples can show one or two unspecific peaks (part 1 of Figure 38, page 73, red and green melting curves). In all cases, a distinct peak must show up in the specific temperature range of the Internal Extraction Control (e.g., part 1 of Figure 38, page 73, blue, red and green melting curves) for valid results.

Absence of a peak in the specific temperature range indicates a negative result of the Internal Extraction Control assay. In this case, the results are invalid, and the extraction must be repeated.
Exceptional case: In case of a specific signal in the sample test and absence of a peak in the Internal Extraction Control, the result of the sample is valid. In this case all PCR controls must perform as expected.

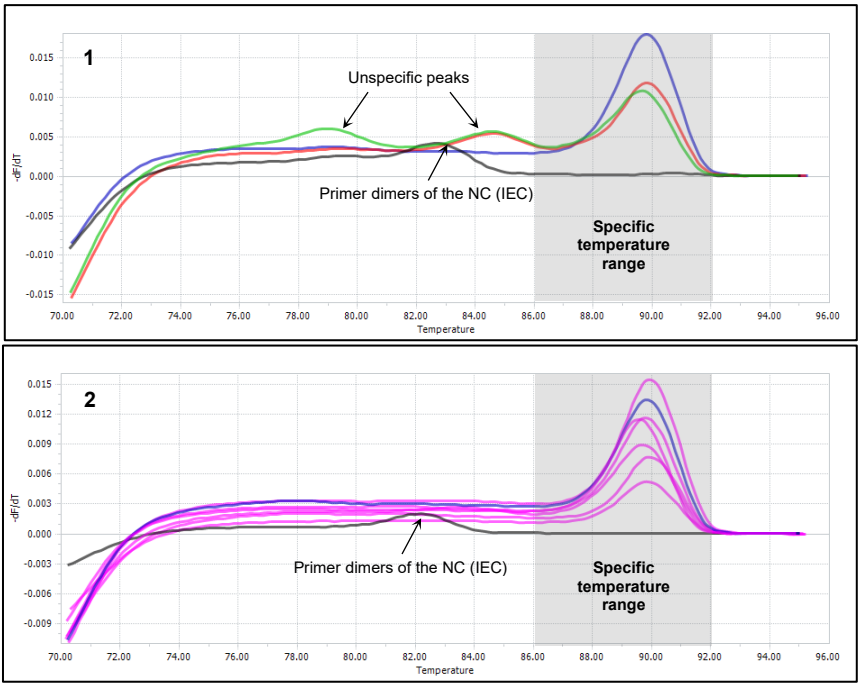
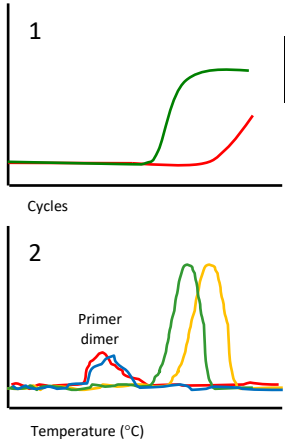


Figure 38: Melting curve analysis (Roche LightCycler® 96) of a negative reference Internal Extraction Control (NC (IEC), black curve) and eluates of samples showing different peaks (colored curves) in assay *MA Control*. The blue sample curve shows a specific peak (90°C) of the Internal Extraction Control. Part 1: The red sample curve shows the specific peak and an unspecific peak (85°C). The green sample curve shows three peaks (specific peak at 90°C and two unspecific peaks at 85°C and 79°C). Part 2: The pink curves show the variety in height of the specific peak. All results are valid.

Schematic Examples for the Interpretation of Real-Time PCR Results:
Examples (A to H, pages 74 to 77) of Real-Time PCRs are shown in a schematic modus of amplification curves (1, upper image) and melting curves (2, lower image). Absolute and relative T_m values can vary among different Real-Time PCR systems. On the right-hand side, the interpretation of the results is given in tables and text. The color code in the table corresponds to the curves in the images. For *MA Control* (IEC and NC IEC) only the melting curve analysis is shown.
In the examples below, it is given that the positive PCR controls (P1 and P2) indicate full functioning of the assay (not illustrated)
Legend to pages 74 to 77:

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.

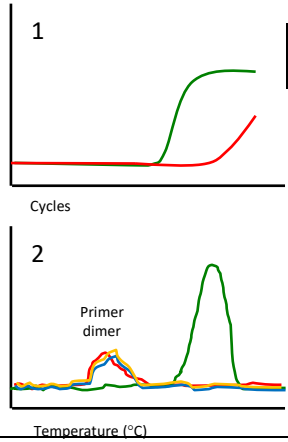
A



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.

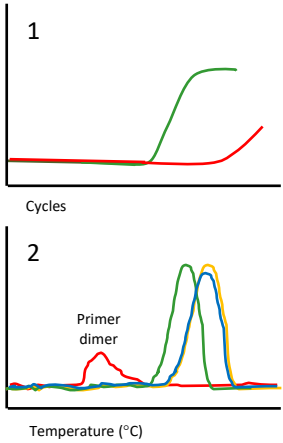
B



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 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



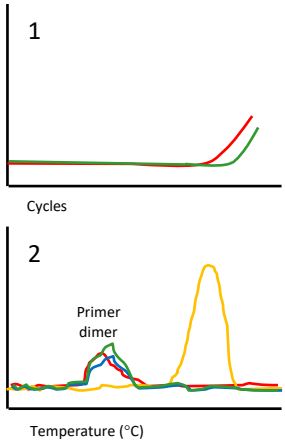
Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
+	-	+	+	+

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



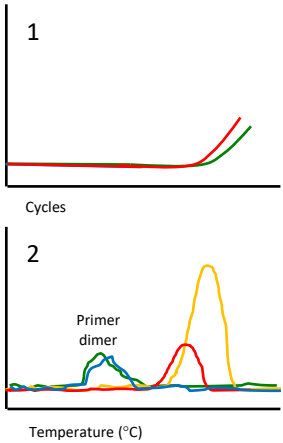
Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
-	-	+	-	-

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



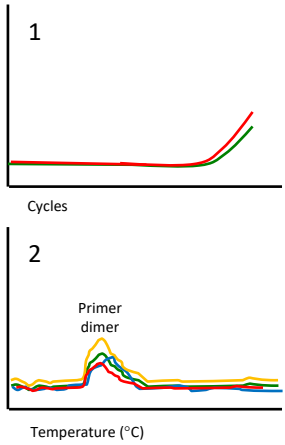
Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
-	+	+	-	-

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



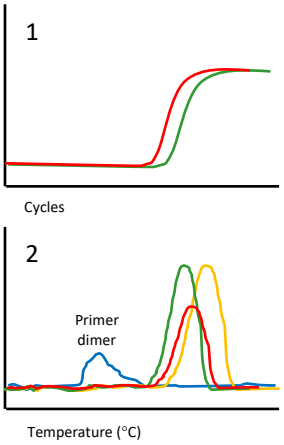
Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
-	-	-	-	?

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 must be repeated.

G



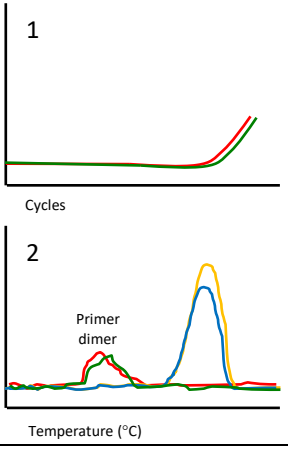
Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
+	+	+	-	?

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 must be repeated with more care to avoid contamination.

H



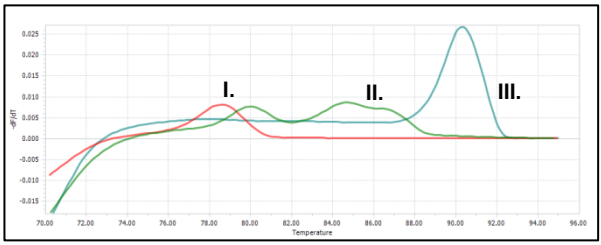
Sample	NC (sample)	IEC	NC (IEC)	Pathogen present?
-	-	+	+	?

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

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



Melting curve analysis (Roche LightCycler® 96) of a negative PCR control (I.), an eluate of a negative sample showing peaks of amplified traces of human DNA (II.) and a positive PCR control showing a specific peak (III.).

Supplementary Information

Troubleshooting

This guide may help solve problems that may arise. For further support:
Phone: +49(0)421 69 61 62 0 • **E-Mail:** support.molzym@bruker.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 (color-code).
	• Enzymes volume too low	Ensure that all enzyme vials are briefly centrifuged before use. Make sure that the enzymes are not frozen when 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 (color-code). Ensure that all enzyme vials are briefly centrifuged before use.
	• PCR inhibition	The *Extraction column* was covered by components such as particles in the sample, washing steps were ineffective 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 mastermixes have been added. Ensure that all reagents (except *MolTaq 16S/18S*) were completely thawed at room temperature and vortexed before use. Make sure that the *MolTaq 16S/18S* is not frozen when pipetting.
False positive PCR result (signal in negative PCR control)	• Cross contamination	Principally, work under UV-irradiated workstations. Avoid the generation of aerosols by carefully 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
	• Contamination during handling	

		different UV workstations (page 22). Clean the vacuum system (section 1F) Cleaning of the vacuum system, page 54). Run cleaning program every two weeks (section 1E) Cleaning script - Cleaning of the pipetting system, pages 52).
	• 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 partially 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 (color-code). Ensure that all enzyme vials are briefly centrifuged before use. In the case of repeated failures at the same sample position in the SelectNA*plus* instrument, contact the technical support for help. 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.
	• Sample still in *Plus-SV* vial	Ensure that the extraction starts with completely filled rows of pipette tips and all following rows are fully filled too. A subsequent check of the selected start tip row of the extraction run can be carried out via the instrument display. For this select 'Services Menu' and choose 'Display Run Log'. Last run numbered with 000 and second last with 001 and so on. The number of samples and the starting row are displayed in the fourth line (right side). In the case of repeated failures at the same sample position in the SelectNA*plus* instrument or run starts correctly with completely filled tip rows, contact the technical support for help.
No eluate	• Process error, e.g., elution buffer on the column	There is no option to subsequently separate the eluate from the finished extraction process. Extraction must be repeated.
	• Rejected sample position	When the extraction program is finished, any rejected sample positions will be shown in the display, when applicable. It shows 'Rejected channel: (position number), see Figure 9 on page 35. Extraction must be repeated. For further information see point 'Rejected sample positions' on page 81.
Eluate volume <70 µl	• The column was partial clogged with remaining particles of the sample (e.g., tissue)	Check carefully the Assay Control (*MA Control*) and repeat the extraction if needed.
Eluate volume >140 µl	• Malfunction of the drying of the column	Check carefully the Assay Control (*MA Control*) and repeat the extraction if needed.

Sample still in *Plus-SV* vial

- Wrong Pipette tip row used as starting point

Ensure that the extraction starts with completely filled rows of pipette tips and all following rows are fully filled too.
A subsequent check of the selected start tip row of the extraction run can be carried out via the instrument display. For this select 'Services Menu' and choose 'Display Run Log'. Last run numbered with 000 and second last with 001 and so on. The number of samples and the starting row are displayed in the fourth line (right side).
In the case of repeated failures at the same sample position in the SelectNA*plus* instrument or run starts correctly with completely filled tip rows, contact the technical support for help.

Error messages

- Error message indicated by an alarm sound when starting the extraction script

The control panel shows e.g., the following error code:
028-007-004-000
013-051-000-000
000-000-000-000
Note the error code and switch off the instrument and start the extraction script 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.
Extraction must be repeated.

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 Figure 9 on page 35 and see Table 7on page 37 for required tip rows per number of samples):

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 two days. For longer storage it is recommended to freeze the samples with a suitable cryoprotectant at -15 to -25°C. - 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) Preparations for sample loading, pages 28 to 31). 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) Preparations for sample loading, pages 28 to 31). Use of inappropriate sample material (see section Product Use Limitations, page 8). Only use the validated specimens listed in Table 3 (page 8).

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Tradenames

Tradename	Factory
ABI 3730XL® and ABI Prism310®	Applied Biosystems
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® 96, 480 and Nano	Roche
Mastercycler®	Eppendorf
Meliseptol® New Formula	B. Braun
Mx3000P® and Mx3005P®	Stratagene
peqStar 96Q	peqlab
QiaQuick®	Qiagen
Reliant®	Lonza
RipSeq®	Pathogenomix
Rotor-Gene®	Qiagen
S-Monovette®	Sarstedt
SYBR® Green1	Invitrogen

Technical Support

If you have questions please call or e-mail us.

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

Material safety data sheets are available on request.

Technical Service

The maintenances of the SelectNA*plus* 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	**24** reactions **48** reactions Automated pathogen DNA isolation and PCR detection.	U-200-024 U-200-048
SelectNAplus 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*

Product	Contents and Application	Cat. No.
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

Order Hotline:

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