Ultra-Deep Microbiome Prep

Depletion of Host DNA, Enrichment and Extraction of Microbial DNA for Next-Generation Sequencing and other Applications

Extraction of
bacterial and fungal DNA from:

Body fluids

Small size sample volumes (≤ 1 ml)
(*ascites, BAL, blood, CSF, pleural fluid, pus, synovial fluid*)

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

DNA-free reagents and consumables

- For *research-use-only* -
- Not for use in diagnostic procedures -

molzym
reveal DNA

Version 07

Date of first release: 01/2013
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Contents

Kit Information

Kit Contents – *Ultra-Deep Microbiome Prep*

	25 rxn (G-020-025)	50 rxn (G-020-050)
Kit 1 – Buffers & Consumables (store at +18 to +25°C)		
CM	1x 12.5 ml	1x 12.5 ml
DB1	1x 12.5 ml	1x 12.5 ml
RS	1x 25 ml	1x 50 ml
RL	1x 5 ml	1x 5 ml
RP	1x 7.5 ml	1x 7.5 ml
CS	1x 12.5 ml	1x 12.5 ml
AB	1x 12.5 ml	1x 12.5 ml
WB	1x 20 ml	1x 20 ml
70% Ethanol, DNA-free	1x 20 ml	1x 20 ml
Deionized water, DNA-free	1x 5 ml	1x 5 ml
TSB	1x 25 ml	2x 25 ml
PKB	1x 7.5 ml	2x 7.5 ml
SU	1x 25 ml	1x 50 ml
ST - Sample tubes, 2.0 ml	1x 50	1x 50
SC - Spin columns in 2.0 ml *Collection tubes*	1x 25	1x 50
CT - Collection tubes, 2.0 ml	1x 50	2x 50
ET - Elution tubes, 1.5 ml	1x 25	1x 50
Kit 1 – Manual		
Manual	1x	1x
Short manual sheets	4x	4x
Kit 2 – Enzymes & Reagents (store at -15 to -25°C), in white box		
MolDNase B, solution	1x 0.25 ml	1x 0.5 ml
BugLysis, solution	1x 0.5 ml	1x 1.0 ml
β-mercaptoethanol, solution	1x 0.08 ml	1x 0.08 ml
Proteinase K, solution	1x 1.0 ml	2x 1.0 ml

Symbols

Symbols used in labelling and in section 'Hazard and Precautionary Statements' (see pages 6 to 8).


Content of the package

REF Catalogue number


Irritant


Manufactured by

Cont. Consult instructions for use


Health hazard


Use by


Flammable


Environmentally Hazardous


Temperature limitation (store at)


Corrosive

LOT Batch code


Toxicity

Storage and Stability

Guarantee for full performance of ***Ultra-Deep Microbiome Prep*** as specified in this manual is only valid if storage conditions are followed. Please take care that the vials of Kit 2 (Enzymes & Reagents) have to be stored at -15 to -25°C upon delivery. Buffers and consumables of Kit 1 should be stored dry in the dark and at room temperature (+18 to +25°C).

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.

Product Use Limitations

Ultra-Deep Microbiome Prep is intended as a kit for ***research-use-only*** and ***not for use in diagnostic procedures.***
Cells must be intact for reliable results. This requires specimens not to be stored in solutions, which induce cell lysis.
The kit is not suitable for frozen samples, if stored without cryoprotectant, as freeze-thaw can cause lysis of microbial cells.

Apparatuses and Consumables to be Supplied by the User

Equipment, consumables and reagents not supplied with this kit have been evaluated by Molzym and are recommended to be used with ***Ultra-Deep Microbiome Prep***.

Sample Preparation:

- 1x thermomixer (24x 2.0 ml tubes), e.g., Eppendorf comfort, Eppendorf, Germany
- 1x vortexer, e.g., VWR, Germany
- 1x bench top microcentrifuge (≥12,000x*g*), e.g., miniSpin, Eppendorf, Germany
- 1x UV Class II biological safety cabinet to be used for the handling of potentially infectious material and extraction of DNA from samples.
- 1x cooling rack for 1.5 ml tubes (-15 to -25°C)
- Sample racks
- Sample positive control (run control):
  BioBall® MultiShot 550 KBE, bioMérieux, Germany
  - BioBall® MultiShot *Candida albicans* NCPF 3179 (56003)
  - BioBall® MultiShot *Escherichia coli* NCTC 12923 (56006)
  - BioBall® MultiShot *Staphylococcus aureus* NCTC 10788 (56009)
- 1 set of precision pipettes: up to 10 µl, 20 µl, 100 µl, 200 µl and 1000 µl, e.g., Eppendorf, Germany
- Sterile forceps (only tissue protocol)
- Sterile support, e.g., Petri dish (only tissue protocol)
- Sterile scalpel or sterile preparation scissors (only tissue protocol)

Plastic Consumables and Reagents

- Pipette tips (with aerosol filter), Biosphere®, Sarstedt, Germany
  - 10 µl type Eppendorf (70.1114.210)
  - 100 µl type Eppendorf (70.760.212)
  - 300 µl type Eppendorf (70.3040.255)
  - 1000 µl type Eppendorf (70.3050.255)
- 1.5 ml micro tubes, Biosphere®, Sarstedt, Germany (72.706.200)
- DNA decontamination, e.g., DNA Exitus®, Applichem, Germany (A7089,0100)
- Sterile disposables
  - Lab coat, e.g., VWR, Germany
  - Gloves, e.g., Kimberly-Clark, Germany
  - Sleeves, e.g., Cardinal Health, Ireland
  - Bouffant Covers, e.g., VWR, Germany
- Waste containers for plastics and liquid waste, autoclavable

Safety Information

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

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

Buffers *CM* and *CS* contain guanidine hydrochloride and guanidinium thiocyanate, respectively, which can form highly reactive compounds and toxic gases when combined with hypochlorite or other acidic solutions. If liquid containing these buffers is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 70% (v/v) ethanol.

This kit is to be used only by skilled personnel trained for handling infectious material. 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. Dispose potentially infectious material and the waste of the sample preparation according to the national directive of the health organization.

Hazard and Precautionary Statements

Buffer *CM*

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

Warning

Hazard and precautionary statements*(page 8):
H302-H315-H319; P301+P312-P302+P352-P305+P351+P338

ß-mercaptoethanol

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

Danger

Hazard and precautionary statements*(page 8):
H301+H331-H310-H315-H317-H318-H361d-H373-H410;
P273-P280-P301+P310-P302+P352+P310-P304+P340+P310-P305+P351+P338

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

Danger

Hazard and precautionary statements*(page 8):
H317-H334; P280-P302+P352-P333+P313-P363

Buffer RP / Buffer PKB
Contains sodium dodecyl sulfate (< 10 %):
Acute toxicity (oral, inhalation), irritation (skin and eye)

Warning

Hazard and precautionary statements*(page 8):
H302-H315-H319-H332; P280-P301+P312-P304+P340+P312-P305+P351+P338

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

Danger

Hazard and precautionary statements*(page 8):

H302-H312-H314-H318-H412-EUH032;
P280-P303+P361+P353-P305+P351+P338-P310-P362+P364

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

Danger

Hazard and precautionary statements*(page 8):
H225-H319-H336; P210-P233-P305+P351+P338

Buffer *WB*
Contains isopropanol (≥ 40 %): **Flammable liquids and irritating (eyes)**.

Danger

Hazard and precautionary statements*(page 8):
H225-H319-H336; P210-P233-P305+P351+P338

70% Ethanol, DNA-free
Contains ethanol (> 50 %): **Flammable liquids and irritating (eyes).**

Danger

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

Emergency Information (24-hours service)

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

* **H225:** Highly flammable liquid and vapour; **H302:** Harmful if swallowed; **H310:** Fatal in contact with skin; **H312:** Harmful in contact with skin; **H314:** Causes severe skin burns and eye damage; **H315:** Causes skin irritation; **H317:** May cause an allergic skin reaction; **H318:** Causes serious eye damage; **H319:** Causes serious eye irritation; **H332:** Harmful if inhaled. **H334:** May cause allergy or asthma symptoms or breathing difficulties if inhaled; **H336:** May cause drowsiness or dizziness; **H361d:** Suspected of damaging fertility. Suspected of damaging the unborn child; **H373:** May cause damage to organs (liver, heart) through prolonged or repeated exposure if swallowed; **H301+H331:** Toxic if swallowed or if inhaled.**H410:** Very toxic to aquatic life with long lasting effects; **H412**: Harmful to aquatic life with long lasting effects; **EUH032**: Contact with acids liberates very toxic gas.

P210: Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking.; **P233:** Keep container tightly closed; **P273:** Avoid release to the environment; **P280:** Wear protective gloves/protective clothing/eye protection/face protection; **P310:** Immediately call a POISON CENTER/doctor; **P363:** Wash contaminated clothing before reuse; **P301+P310:** IF SWALLOWED: Immediately call a POISON CENTER or doctor; **P301+P312:** IF SWALLOWED: Call a POISON CENTER/ doctor if you feel unwell.; **P302+P352:** IF ON SKIN: Wash with plenty of water; **P302+P352+P310:** IF ON SKIN: Wash with plenty of water. Immediately call a POISON CENTER/doctor; **P303+P361+P353:** IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water [or shower]; **P304+P340+P310:** IF INHALED: Remove person to fresh air and keep comfortable for breathing. Immediately call a POISON CENTER/doctor; **P304+P340+P312:** IF INHALED: Remove person to fresh air and keep comfortable for breathing. Call a POISON CENTER/doctor if you feel unwell; **P305+P351+P338:** IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing; **P362+P364:** Take off contaminated clothing and wash it before reuse.

Kit Description

The *Ultra-Deep Microbiome Prep* Procedure

In its concept, ***Ultra-Deep Microbiome Prep*** is a pre-analytical means of molecular analysis of microbial communities from a variety of body sites.
Ultra-Deep Microbiome Prep combines new solutions for DNA extraction from ≤ 1 ml fluid samples (e.g., ascites, BAL, CSF, synovial fluid) and tissue samples (e.g., abscesses, biopsies, native/prosthetic heart valves). The procedure includes the depletion of host DNA (≥ 95 %) and the extraction of DNA from bacteria and fungi from clinical specimens for Next-Generation Sequencing and other applications.

Sample Extraction

Next-Generation Sequencing research approaches demand high resolution of microbial communities colonizing the human body. To gain a comprehensive image of communities factors negatively influencing the analysis should be avoided as far as possible (Fig. 1).

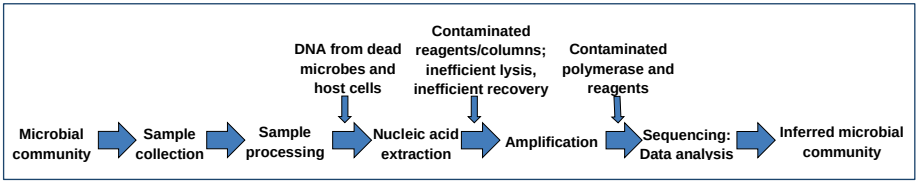

Fig. 1: Steps in the processing of samples for Next-Generation Sequencing analysis (adapted from Rogers & Bruce, 2010) and pre-analytic parameters negatively influencing the results. Thereby, the inferred microbial community structure and function may be significantly divergent from the real microbial community.

Among these factors are irrelevant sequences intrinsic to and brought into specimens. In particular, a major factor is human DNA and extracellular DNA which usually outnumbers the microbial target DNA and which in broad-range amplification approaches are co-amplified with target sequences from living microbes. A solution is desirable to remove irrelevant non-target DNA already at the level of extraction. Also, because microbial communities can consist of hundreds of species belonging to very diverse taxa, the extraction procedure must guarantee availability of DNA from optimally any microbe present in a sample. Last, the input of microbial DNA into sample analysis by contaminated extraction and amplification reagents can bias the analysis. Therefore, reagents have to be DNA-free. To all of these problems ***Ultra-Deep Microbiome Prep*** contributes by supplying innovative solutions.

The analysis of specimens using ***Ultra-Deep Microbiome Prep*** follows a sequence of steps (see **Fig. 2**, page 10). Microbial DNA extraction is initiated by treatment of the sample with a buffer that lyses human / animal cells followed by a DNase treatment for the degradation of free floating DNA, including DNA from lysed human / animal cells and extracellular DNA. Microbial cells are then concentrated by centrifugation and exposed to a reagent, *BugLysis*, that degrades the cell walls of Gram-positive and Gram-negative bacteria and fungi. *Proteinase K* treatment finalizes the sample extraction which is followed by a bind-wash-elute spin column DNA purification protocol.

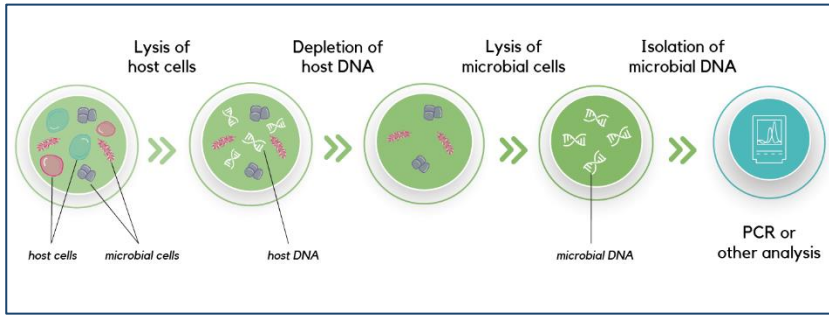

Fig. 2: The principle of enrichment and extraction of microbial DNA from specimens by *Ultra-Deep Microbiome Prep.*

Various NGS technologies and platforms enable a wide variety of applications to address specific research objectives. Whole genome sequencing and targeted re-sequencing approaches require highest quality target DNA in profiling microbial communities associated with the human body at high resolution. Samples normally contain complex DNA mixtures with high amount of human DNA and only a fractional part of microbial DNA which is also depending on the specimens (Fig. 3). The pre-analytical reduction of human DNA load can improve the output of sequence reads in a clinical sample.

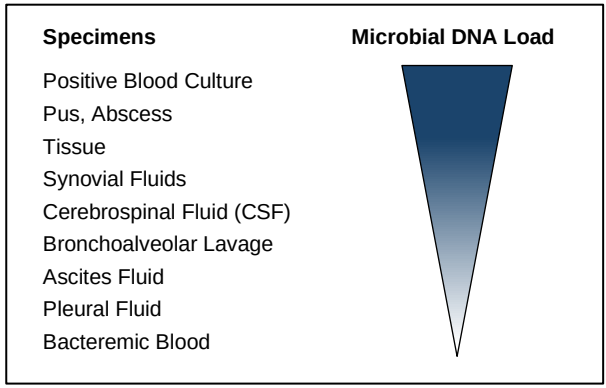

Fig. 3: Microbial DNA loads of a variety of specimens. Full-grown blood cultures can contain >10^9 cfu/ml, while bacteremic blood usually contains < 10-100 cfu/ml.

Controls and Validation

Controls

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

Sample Controls:

Positive sample control (run control)
This control reflects the performance of the extraction procedure and should be performed at least once per setup. There are two ways suggested to perform a run control:
i) Negative samples (e.g., buffer *SU*) are spiked with 100 to 1000 cfu/ml of a cultured Gram-negative (e.g., *E. coli*), Gram-positive (e.g., *S. aureus*) and fungal (e.g., *C. albicans*) pathogen, respectively, and run through the extraction protocol followed by PCR analysis (e.g., Molzym´s DNA-free PCR reagents).
ii) The extraction is performed using a commercial standard, e.g., BioBall® MultiShot 550 KBE (bioMérieux, Germany).

Negative sample control
This test should be run together with the sample positive control to test for potential cross-contamination during sample extraction. For this, a negative sample (buffer *SU*) is used and run through the extraction of this kit.

PCR Controls:

Internal PCR control
Potential inhibition of the PCR reaction by sample components co-extracted with DNA can be measured by an internal control assay (not included in this product).

Validation

Broad-range lysis of microorganisms

The broad-range lysis capacity of ***Ultra-Deep Microbiome Prep*** for Gram-positive and Gram-negative bacteria, and fungi has been demonstrated in a variety of clinical evaluations, including various fluid and tissue specimens.

The broad-range lysis potential has been shown in clinical evaluations, including over 200 genera of bacteria (86 Gram-positives, 120 Gram-negatives) and 65 genera of fungi (Tab. 1).

Tab. 1: Extract of microorganisms identified in clinical evaluations. Full list available at www.molzym.com.

Gram-negative bacteria	*Helicobacter pylori*	*Alloiococcus otitis*	*Nocardia* spp.
Achromobacter xylosoxidans	*Kingella* spp.	*Anaerococcus* spp.	*Paenibacillus* spp.
Acidovorax spp.	*Klebsiella* spp.	*Atopobium* spp.	*Parvimonas micra*
Acinetobacter spp.	*Kerstersia* spp.	*Bacillus* spp.	*Peptoniphilus* spp.
Aeromonas veronii	*Kluyvera cryocrescens*	*Bifidobacterium* spp.	*Peptostreptococcus* spp.
Afipia broomeae	*Lautropia mirabilis*	*Brevibacterium* spp.	*Propionibacterium* spp.
Aggregatibacter aphorophilus	*Legionella pneumophila*	*Carnobacterium* spp.	*Rhodococcus* spp.
Anaerotruncus colihominis	*Leptotrichia* spp.	*Clostridium* spp.	*Rothia* spp.
Bacteroides spp.	*Massilia* spp.	*Coprococcus catus*	*Staphylococcus* spp.
Bartonella quintana	*Methylobacterium* spp.	*Corynebacterium* spp.	*Streptococcus* spp.
Bordetella spp.	*Moraxella* spp.	*Dermabacter hominis*	*Tropheryma whippleii*
Borrelia garinii	*Morganella morganii*	*Dietzia* spp.	*Tsukamurella* spp.
Bosea spp.	*Neisseria* spp.	*Dolosigranulum pigrum*	*Ureaplasma urealyticum*
Brucella spp.	*Pantoea agglomerans*	*Eggerthella lenta*	*Vagococcus* spp.
Burkholderia spp.	*Paracoccus* spp.	*Enterococcus* spp.	*Wolbachia* spp.
Campylobacter spp.	*Pasteurella* spp.	*Eremococcus coleocola*	
Candidatus Neoehrlichia mikurensis	*Porphyromonas* spp.	*Eubacterium* spp.	**Fungi**
Capnocytophaga spp.	*Prevotella* spp.	*Facklamia* spp.	*Aspergillus* spp.
Chryseobacterium indologenes	*Proteus* spp.	*Finegoldia magna*	*Candida* spp.
Citrobacter freundii	*Providencia stuartii*	Gardnerella vaginalis	*Cladosporium cladosporioides*
Cloacibacterium normanense	*Pseudomonas* spp.	*Gemella* spp.	*Cryptococcus* spp.
Comamonas testosteroni	*Ralstonia* spp.	*Gordonia* spp.	*Didymella exitialis*
Coxiella burnetii	*Raoultella planticola*	*Granulicatella adiacens*	*Davidiella tassiana*
Cronobacter sakazakii	*Rickettsia typhi*	*Janibacter* spp.	*Fusarium* spp.
Curvibacter spp.	*Serratia marcescens*	*Kocuria* spp.	*Issatchenkia orientalis*
Delftia spp.	*Shigella* spp.	*Lactobacillus* spp.	*Malassezia* spp.
Dialister spp.	*Stenotrophomonas maltophilia*	*Lactococcus* spp.	*Pseudallescheria boydii*
Elizabethkingia meningoseptica	*Veillonella* spp.	*Leifsonia* spp.	*Saccharomyces cerevisiae*
Enhydrobacter aerosaccus	*Weeksella* spp.	*Listeria monocytogenes*	*Schizophyllum radiatum*
Enterobacter spp.	*Yersinia* spp.	*Microbacterium* spp.	*Sporobolomyces* spp.
Escherichia spp.	**Gram-positive bacteria**	*Micrococcus* spp.	
Fusobacterium spp.	*Abiotrophia* spp.	*Mogibacterium timidum*	**Protist**
Haemophilus spp.	*Actinomyces* spp.	*Mycobacterium* spp.	*Plasmodium* spp.
Hafnia alvei	*Aerococcus* spp.	*Mycoplasma* spp.	

Avoidance of DNA Contamination

Care for the avoidance of DNA contamination from exogenous sources includes the complete pathway from sample collection to analysis. Also, it is important to minimize cross-contamination from sample to sample as far as possible. A short summary of precautions is given below:

- Generally, for pre-analytical and analytical processing, use places decontaminated from DNA. We recommend to perform handling steps under UV-irradiated workstations. UV irradiation must be done before working according to the guidelines of the manufacturer. Routinely treat the surfaces of the working places with a commercial DNA decontamination reagent which is compatible with protective gloves. Make sure that the material to be decontaminated is resistant to such treatment. Do not transfer supplies (e.g., pipettes, microcentrifuges, vortexer) and disposable material as specified by the handlings below from one working place to another. Each working place should be equipped with refrigerators (+4 to +12°C) and freezers (-15 to -25°C) for storage of the reagents of the kit.
- Handle potentially infectious material with great care under a UV Class II biological safety cabinet in order to protect yourself from infection, and to avoid cross-contamination of samples and carry-over contamination of extraction buffers and reagents.
- Wear sterile protective gloves and sterile disposable sleeve covers at any handling step, including handling of potentially infectious material, DNA preparation and PCR analysis. Frequently change protective gloves during handling. Use disposable lab coats, protective goggles and disposable overshoes and change when moving from one laboratory to another.
- Take care to maintain a DNA-free environment during opening the vials and bottles. Close vessels immediately after the removal of liquid.
- Use only DNA-free pipette tips, vials and consumables recommended (see page 5).

Pre-Analytics

Sample Collection

Special care has to be taken for sample collection and handling to avoid contamination by skin and environmental microorganisms.

Transfer the sample to the laboratory for immediate processing. Alternatively, store the sample in a refrigerator (+4 to +12°C). Make sure that storage does not alter the sample with respect to potential changes in community structure. For longer storage, freezing may be an option. However, bear in mind that microorganisms may tend to be lysed by freeze-thawing. Because of a DNase treatment during sample preparation, this may lead to loss of microbial DNA. Molzym offers solutions for freeze storage of fluid samples.

Contact Molzym for further information:
Tel.: +49(0)421-69 61 62 0 • **E-Mail:** support@molzym.com

Isolation of Microbial DNA

Work in a place, ideally in a lab separated from places where mastermixes are handled and PCR reactions are performed. Calibrate the procedure by spiking negative samples (e.g., blood or sterile buffer) with dilutions of full-grown cultures of microorganisms as advised (see Sample Control, page 11), or by using BioBall® MultiShot 550 KBE (see page 5, Sample Preparation)

Important notes: please read before starting

! Work in a UV Class II biological safety cabinet. The UV lamp must be switched off during working.

! **Body fluid and tissue specimens:** Sampled under aseptic conditions and transferred to a sterile sample container (not supplied).

Whole blood samples: Use only EDTA or citrate-stabilized blood

! For optimal results, use only fresh samples. ***Do not freeze samples*** to avoid loss of microbial DNA due to cell disruption as a result of freezing and thawing and DNase treatment during extraction. Alternatively, the following procedure of microbial DNA isolation can be interrupted (see step 5 in protocols 1 and 2). For longer storage of fluid samples, use Molzym's *UMD-Tubes* (order no. Z-801-020).

! For equipment and consumables to be supplied by the user see page 5.

! Take care that *MolDNase B, BugLysis*, *β-mercaptoethanol* and *Proteinase K solutions* (Kit 2) are placed in a cooling rack adjusted to -15 to -25°C. Replace enzymes and reagent to the freezer (-15 to -25°C) immediately after handling.

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

! Adjust the thermomixer to 37°C (protocol 1) or to 56°C (protocol 2). Pipette an aliquot of *Deionized water*, DNA free (100µl for each sample) into a sterile 1.5 ml micro tube (not supplied) and place into the thermomixer (needed for step 15 in protocols 1 and 2).

! To avoid carry-over contamination, close caps of vials and bottles immediately after removal of solution.

! Leave items used for the workflow (e.g., pipettes, racks, pipette boxes) in the workstation and expose them to the UV irradiation for decontamination before starting. In case of contamination of pipettes and other items or spilling the surface of the workstation with sample material decontaminate as advised (see Safety Information, page 6). Arrange all items according to your personal customs.

DNA Extraction and Purification Protocols

Caution: Wear sterile protective gloves, sterile disposable sleeve covers, a lab coat, protective goggles and bouffant cover when handling potentially infectious material. Work in a laminar flow workstation 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.

How to Start

Kit 1 contains buffers and consumables for the extraction and isolation of microbial DNA. Open the bottles and bags only in the Class II biological safety cabinet, remove the consumables needed from each bag. Arrange bottles according to the sequence of steps as below:

SU (protocol 1) or PKB – TSB (protocol 2)

CM – DB1 – RS – RL – RP – CS – AB – WB – 70% Ethanol – Deionized water

Two protocols are supplied: One for fluid samples ≤ 1 ml (protocol 1) and another for tissue samples (protocol 2).

Protocol 1: Fluid Samples
(e.g., ascites, BAL, blood, CSF, pleural fluid, pus, synovial fluid)

A) Fill up procedure for samples less than 1 ml volume

Samples less than 1 ml are filled up using buffer *SU*. Transfer the sample into a supplied sterile 2.0 ml *Sample tube* (*ST*, Kit 1). Then add buffer *SU* until reaching the 1 ml mark of the tube. Discard pipette tip with excess buffer *SU*. Never re-use any pipette tip after use. Continue with part B (below).

B) Sample pre-treatment and DNA isolation procedure

1. **Pipette 1 ml sample into a sterile 2 ml *Sample tube* (*ST tube,* Kit 1) or use filled-up sample (part A, above). Then add 250 µl buffer *CM* and vortex at full speed for 15 s to mix. Let stand on the bench at room temperature (+18 to +25°C) for 5 min.**

   Buffer *CM* is a chaotropic buffer that lyses the human/animal cells. For optimal results it is important to mix thoroughly.

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

2. **Add 250 µl buffer *DB1* and 10 µl *MolDNase B* (do not premix) to the lysate and immediately vortex for 15 s. Let stand at room temperature (+18 to +25°C) for 15 min.**

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

3. **Harvest bacterial and fungal cells by centrifugation in a bench top centrifuge at ≥12,000x*g* for 10 min. Thereafter, carefully remove the supernatant by pipetting.**

   Human cell debris and potentially present microbial cells are sedimented.

4. **Pipette 1 ml buffer *RS* to the sediment and resuspend by vigorous vortexing.**
   Resuspension may take some time. The pellet consists of cell debris and microorganisms. Take care that all visible material has been resuspended before continuing.

5. **Centrifuge the *ST tube* in a bench top microcentrifuge (≥12,000x*g*) for 5 min. Carefully remove the supernatant by pipetting.**
   This washing removes residual *MolDNase B* activity, chaotrope and most of the PCR inhibitors. The sediment contains potentially present bacteria and fungi.

   At this point the procedure can be interrupted by freezing the sample (-15 to -25°C). For further processing, thaw the sample to room temperature (+18 to +25°C) and proceed with step 6, page 17.

6. **Pipette 80 µl buffer *RL* to the *ST tube*. Resuspend the sediment by stirring with a pipette tip and pipetting in and out for several times.**
7. **Pipette 20 µl *BugLysis* and 1.4 µl *ß-mercaptoethanol* directly into the extract. Vortex the tube for 15 s and incubate in a thermomixer at 37°C and 1,000 rpm for 30 min.**

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

   Caution: ß-mercaptoethanol is toxic. Take care not to inhale and otherwise come into contact with.
8. **Adjust the temperature of the thermomixer to 56°C. Add 150 µl buffer *RP* and 20 µl *Proteinase K* to the *ST tube*. Vortex at full speed for 15 s and incubate at 56°C and 1,000 rpm for 10 min.**
9. **Briefly centrifuge the *ST tube* and pipette 250 µl buffer *CS* into it. Vortex tube at full speed for 15 s.**

   Cells are lysed and protein is denatured.
10. **Briefly centrifuge and add 250 µl binding buffer *AB* to the *ST tube*. Vortex at full speed for 15 s.**
11. **Briefly centrifuge and transfer the lysate to a *Spin column* (*SC*; Kit 1) by pipetting. Close the lid of the *SC column* and centrifuge at ≥12,000x*g* for 30 s (or minimum time of the centrifuge, e.g. 60 s).**

    At this point nucleic acids bind to the matrix.
12. **Remove the *SC column* and place into another 2 ml *Collection tube* (*CT*; Kit 1). Discard the *CT tube* containing the flow-through. Pipette 400 µl buffer *WB* to the *SC column*. Close the lid and centrifuge at ≥12,000x*g* for 30 s (or minimum time of the centrifuge, e.g. 60 s).**
13. **Remove the *SC column* and place into another 2 ml *CT tube*. Wash the *Spin column* with 400 µl of *70% Ethanol* by centrifugation at ≥12,000x*g* for 3 min.**

    This step removes salts and dries the column matrix.
14. **Carefully remove the closed *SC column* from the centrifuge. Avoid splashing of the flow-through to the *SC column* because ethanol is a PCR inhibitor. Remove the *SC column* from the *CT tube* and place into a sterile 1.5 ml *Elution tube* (*ET*; Kit 1).**
15. **Pipette 100 µl of *Deionized water* (pre-heated to 70°C) in the center of the *SC column*. Close the lid and incubate at room temperature (+18 to +25°C) for 1 min. Thereafter, centrifuge at ≥12,000x*g* for 1 min to elute the DNA. Finally, remove the *SC column* from the *ET tube* and close the lid. Discard the *SC column*.**
16. **Store the *ET tube* containing the eluate at +4 to 12°C if analyzed at the same day 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).

Protocol 2: Tissue Samples
(e.g., abscesses, biopsies, heart valves, prostheses, stents)

A) Pre-Treatment of Tissue Samples

- **Tissue specimens are sampled under conditions avoiding contamination and transported to the laboratory.**
- **Pipette 180 µl of buffer *PKB* (Kit 1) into a *Sample tube* (*ST* tube, Kit 1).**
- **Transfer the specimen to a sterile support, e.g., a Petri dish, by using sterile forceps. For preparation of the tissue specimen, the area should measure at maximum approx. 0.5x 0.5 x 0.5 cm. Cut the specimen into small pieces by using a sterile scalpel or sterile preparation scissors. Thereafter, transfer the cut specimen to the *ST* tube filled with buffer *PKB*. Add 20 µl of *Proteinase K* (Kit 2) to the specimen.**
- **Vortex the *ST* tube at full speed for 15 s and incubate in the thermomixer at 56°C and 1,000 rpm for 10 min.**

  After the incubation, adjust the thermomixer to 37°C.

  Comment: The tissue is partially digested and may decay. Potentially present bacteria and fungi are released from biofilms.
- **Fill up to 1 ml with the transport solution, if available, or with buffer *TSB* (use the measure line of the tube). Continue with part B (below).**

B) Sample pre-treatment and DNA isolation procedure

1. **Pipette 250 µl buffer *CM* to the *ST tube*. Vortex at full speed for 15 s to mix. Let stand at room temperature (+18 to +25°C) for 5 min.**

   Buffer *CM* is a chaotropic buffer that lyses human/animal cells. For optimal results it is important to mix thoroughly.

   Caution: buffer *CM* is an irritant. Avoid contact with skin and eyes.
2. **Briefly centrifuge to clear the lid. Pipette 250 µl buffer DB1 to the *ST tube*. Thereafter, pipette 10 µl *MolDNase B* to the lysate in the *ST tube*. Immediately vortex for 15 s and let stand for 15 min at room temperature (+18 to +25°C).**

   During this step the nucleic acids released from human cells and extracellular DNA are degraded.
3. **Centrifuge the *ST tube* in a bench top microcentrifuge at ≥12,000x*g* for 10 min. Thereafter, carefully remove the supernatant by pipetting and discard.**

   Debris from lysed tissue and potentially present microbial cells are sedimented.
4. **Pipette 1 ml buffer *RS* to the sediment and resuspend by vigorous vortexing.**

   Depending on the specimen, the sediment may contain residues of solid material (debris and undigested tissue) and may be rigid. Resuspension may take some time.
5. **Centrifuge the *ST tube* in a bench top microcentrifuge at ≥12,000x*g* for 5 min. Carefully remove the supernatant by pipetting and discard.**

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

At this point the procedure can be interrupted by freezing the sample (-15 to -25°C). For further processing, thaw the sample to room temperature (+18 to +25°C) and proceed with step 6, page 19.

6. **Pipette 80 µl buffer *RL* to the *ST tube.* Resuspend the sediment by vigorous vortexing or by stirring with a pipette tip and pipetting in and out for several times. Briefly centrifuge to clear the lid.**
7. **Pipette 20 µl *BugLysis* and 1.4 µl *ß-mercaptoethanol* (Kit 2) directly into the extract. Vortex the tube for 15 s and incubate in a thermomixer at 37°C and 1,000 rpm for 30 min.**

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

   Caution: ß-mercaptoethanol is toxic. Take care not to inhale and otherwise come into contact with.
8. **Adjust the temperature of the thermomixer to 56°C. Add 150 µl buffer *RP* and 20 µl *Proteinase K* to the *ST tube*. Vortex at full speed for 15 s and incubate at 56°C and 1,000 rpm for 10 min.**
9. **Briefly centrifuge the *ST tube* and pipette 250 µl buffer *CS* into it. Vortex tube at full speed for 15 s.**

   Microbial cells are lysed and protein is denatured.
10. **Briefly centrifuge and add 250 µl binding buffer *AB* to the *ST tube*. Vortex at full speed for 15 s.**
11. **Briefly centrifuge and transfer the lysate to a *Spin column* (*SC*; Kit 1) by pipetting. *Do not transfer potentially present undigested material (pulse centrifuge to sediment and pipette only the supernatant).* Close the lid of the *SC column* and centrifuge at ≥12,000x*g* for 30 s (or minimum time of the centrifuge, e.g. 60 s).**

    At this point nucleic acids bind to the matrix.
12. **Remove the *SC column* and place into another 2 ml *Collection tube* (CT; Kit 1). Discard the *CT tube* containing the flow-through. Pipette 400 µl buffer *WB* to the *SC column*. Close the lid and centrifuge at ≥12,000x*g* for 30 s (or minimum time of the centrifuge, e.g. 60 s).**
13. **Remove the *SC column* and place into another 2 ml *CT tube* (CT; Kit 1). Discard the *CT tube* containing the flow-through. Wash the SC column with 400 µl *70% Ethanol* by centrifugation at ≥12,000x*g* for 3 min.**

    This step removes salts and dries the column matrix.
14. **Carefully remove the closed *SC column* from the centrifuge. Avoid splashing of the flow-through to the *SC column* because ethanol is a PCR inhibitor. Remove the *SC column* from the *CT tube* and place into a sterile 1.5 ml *Elution tube* (ET; Kit 1).**
15. **Pipette 100µl *Deionized water* (pre-heated to 70°C) in the center of the *SC column*. Close the lid and incubate at room temperature (+18 to +25°C) for 1min. Thereafter, centrifuge at ≥12,000x*g* for 1min to elute the DNA. Finally, remove the *SC column* from the *ET tube* and close the lid. Discard the *SC column*.**
16. **Store the *ET tube* containing the eluate at +4 to 12°C°C if analyzed at the same day 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).

Supplementary Information

Troubleshooting

This guide may help solve problems that may arise. For further support:
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Observation	Possible cause	Comments/suggestions
Strong human/animal DNA background	• Buffer *CM* not added • Buffer *DB1* not added • *MolDNase B* not added • Solution not mixed	Eluates usually contain traces of human DNA co-eluted with microbial DNA. If the extraction has not been performed according to the protocol, increased amounts of human DNA can be the result, which negatively influences the analysis. Ensure that buffer *CM* has been added to lyse human cells. Accordingly, addition of buffer *DB1* and *MolDNase B* is obligate. Keep the *MolDNase B* vial chilled, because warming may reduce enzyme activity and hence increase human DNA background. It is important that solutions are thoroughly mixed after addition of buffers. Follow instructions for vortexing.
False negative result (no signal in control PCR assay)	• PCR inhibitors co-eluted	Check whether *Proteinase K* treatment has been performed during DNA preparation. Make sure that all washing steps of the procedure have been followed. Optionally, after *70% Ethanol* washing (step 14 of protocols 1 and 2), centrifuge for another 1 min to avoid ethanol carry over to the eluate.
False positive result (signal in negative control)	• Cross-contamination • Contamination during handling	Principally, work under UV-irradiated workstations. Avoid the generation of aerosols by careful pipetting. Open vials and tubes only shortly for pipetting and close again immediately thereafter. Frequently change gloves. UV-irradiate the workstation at the end of handling a series of samples. Prepare mastermixes and handle DNA samples under different UV workstations. Use DNA-free filter pipette tips and other plastics only as recommended (see page 5).

Observation	Possible cause	Comments/suggestions
No amplicon detectable	• Insufficient lysis • PCR inhibition	Make sure that *BugLysis* has been added. Ensure that the *Proteinase K* treatment has been performed. Run the internal control assay (not included) for testing for potential PCR inhibition. Check whether the amplification conditions are optimal with regard to primer annealing, reaction times and cycle numbers.
	• Insufficient homogenization • Microbe number too low	If the sediments at steps 3 and 5 of protocols 1 and 2 are not resuspended, microbial cells may be included in the debris and not reached by lytic enzymes. To increase the sensitivity of the assay, increase the volume of eluate in the PCR reaction (up to 5µl per 25µl run).
	• Loss of nucleic acids during purification	Ensure that buffer *AB* has been added to and mixed with the lysate (step 10 of protocols 1 and 2).
	• Suboptimal elution conditions	Make sure to elute with heated DNA-free water (70°C; step 15 of protocols 1 and 2). This increases the DNA yield significantly.
	• Loss of nucleic acids during the storage of the eluate	Store the eluted DNA at +4 to +12°C if analysed at the same day 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).

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Information DNA-Free PCR Reagents

A common drawback of PCR assays targeting microbial sequences is the contamination of amplification reagents by microbial DNA. This problem becomes even more evident when the assay is directed to a broad range of bacterial and fungal targets. Consequences of DNA contamination may be false-positive results and loss in analytical sensitivity.

Molzym´s Mastermix 16S/18S products are guaranteed free of contaminating DNA thus generating reliable results.

Product	Contents	Cat. No.
DNA-free PCR reagents		
Mastermixes, DNA-free (2.5x concentrated)		
Mastermix 16S Complete Universal 16S rDNA PCR and Real-Time PCR assay for detection of bacterial DNA	100 reactions 250 reactions 1000 reactions	S-020-0100 S-020-0250 S-020-1000
Mastermix 18S Complete Universal 18S rDNA PCR and Real-Time PCR assay for detection of fungal DNA	100 reactions 250 reactions 1000 reactions	S-070-0100 S-070-0250 S-070-1000
Mastermix 16S/18S Dye Premixed reagents and fluorescent dye for Real-Time PCR with custom primers	100 reactions 250 reactions 1000 reactions	S-030-0100 S-030-0250 S-030-1000
Mastermix 16S/18S Basic Premixed reagents for PCR analysis with custom primers	100 reactions 250 reactions 1000 reactions	S-040-0100 S-040-0250 S-040-1000
Taq DNA Polymerase, DNA-free		
MolTaq 16S/18S	100 units 500 units	P-019-0100 P-019-0500
Hot MolTaq 16S/18S	100 units 500 units	P-080-0100 P-080-0500
PCR-Grade Water, DNA-free		
DNA-free water, *PCR grade*	10x 1.7 ml	P-020-0003

Tradenames

Tradename	Factory
BioBall® MultiShot 550 KBE	bioMérieux
Biosphere®	Sarstedt
Hot MolTaq 16S/18S	Molyzm
Mastermix 16S Complete	Molyzm
Mastermix 18S Complete	Molyzm
Mastermix 16S/18S Basic	Molyzm
Mastermix 16S/18S Dye	Molyzm
MolTaq 16S/18S	Molyzm
MolYsis™ Basic5	Molyzm
MolYsis™ Complete5	Molyzm
MolYsis-SelectNA™*plus*	Molyzm
SelectNA™*plus*	Molzym
Ultra-Deep Microbiome Prep	Molzym
Ultra-Deep Microbiome Prep10	Molzym

Technical Support

If you have questions please contact us.

Our hotline: +49(0)421 69 61 62 0

E-Mail: support@molzym.com

Web: www.molzym.com

Material safety data sheets are available on request.

Order Information

Product	Contents	Cat. No.
Ultra-Deep Microbiome Prep Kit includes reagents for tissue pre-treatment, host DNA depletion, microbial DNA extraction and purification. ≤1 ml fluid samples ≤0.5 cm3 tissue samples	25 reactions 50 reactions	G-020-025 G-020-050

Related Products

Product	Contents	Cat. No.
MolYsis™Basic5 Flexible solution for host DNA depletion from body fluids – to be used with other DNA isolation kits. ≤1ml and 5ml fluid samples	50 reactions 100 reactions	D-301-050 D-301-100
MolYsis™Complete5 Kit includes reagents for host DNA depletion, microbial DNA extraction and purification. ≤1 ml and 5 ml fluid samples	50 reactions 100 reactions	D-321-050 D-321-100
Ultra-Deep Microbiome Prep10 Kit includes reagents for tissue pre-treatment, host DNA depletion, microbial DNA extraction and purification. 1-10 ml fluid samples ≤0.5 cm^3 tissue samples	25 reactions 50 reactions	G-030-025 G-030-050
Automated solution to be used with the SelectNA™*plus* benchtop instrument:		
MolYsis-SelectNA™plus Kit includes reagents for tissue pre-treatment, host DNA depletion, microbial DNA extraction and purification. ≤1 ml fluid samples & swabs ≤0.5 cm^3 tissue samples	48 reactions	D-450-048

See also Molzym's homepage (www.molzym.com) for more information.

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