

Ultra-Deep Microbiome Prep10

**Removal of Host DNA, Enrichment and
Extraction of Microbial DNA
for Next-Generation Sequencing**

Extraction of
bacterial and fungal DNA from:

Body fluids

Large size sample volumes (1 to 10ml)
(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 *in-vitro* diagnostic use -**



© 2013-2020 Molzym, all rights reserved

Version 05

Date of first release: 01/2013

Last update: 10/2020

Contents

| | |
|--|-----------|
| Kit Information | 3 |
| Kit Contents – <i>Ultra-Deep Microbiome Prep10</i> | 3 |
| Symbols | 4 |
| Storage and Stability | 4 |
| Product Use Limitations..... | 4 |
| Apparatuses and Consumables to be Supplied by the User | 5 |
| Safety Information | 6 |
| Hazard and Precautionary Statements..... | 6 |
| Kit Description | 8 |
| The <i>Ultra-Deep Microbiome Prep10</i> Procedure | 8 |
| Sample Extraction | 8 |
| Controls and Validation | 11 |
| Controls..... | 11 |
| Validation | 12 |
| Avoidance of DNA Contamination | 13 |
| Pre-Analytics | 14 |
| Sample Collection | 14 |
| Isolation of Microbial DNA | 14 |
| DNA Extraction and Purification Protocols | 15 |
| How to Start..... | 15 |
| Protocol 1: Liquid Samples | 16 |
| Protocol 2: Tissue Samples | 18 |
| Supplementary Information | 20 |
| Troubleshooting | 20 |
| Tradenames | 22 |
| References..... | 22 |
| Associated Products..... | 23 |
| Technical Support | 24 |
| Order Information | 24 |
| Contact | 24 |














Kit Information

Kit Contents – *Ultra-Deep Microbiome Prep10*

| | 25 rxn | 50 rxn |
|---|-----------|-----------|
| Kit 1 – Buffers & Consumables (store at +18 to +25°C) | | |
| CM | 1x 100ml | 2x 100ml |
| DB1 | 1x 100ml | 2x 100ml |
| RS | 1x 25ml | 1x 50ml |
| RL | 1x 5ml | 1x 5ml |
| RP | 1x 7.5ml | 1x 7.5ml |
| CS | 1x 12.5ml | 1x 12.5ml |
| AB | 1x 12.5ml | 1x 12.5ml |
| WB | 1x 20ml | 1x 20ml |
| 70% Ethanol, DNA-free | 1x 20ml | 1x 20ml |
| Deionized Water, DNA-free | 1x 5ml | 1x 5ml |
| TSB | 1x 25ml | 2x 25ml |
| PKB | 1x 7.5ml | 2x 7.5ml |
| SU | 2x 125ml | 4x 125ml |
| ST - Sample tubes, 2.0ml | 1x 50 | 1x 50 |
| SC - Spin columns in 2.0ml Collection tubes | 1x 25 | 1x 50 |
| CT - Collection tubes, 2.0ml | 1x 50 | 2x 50 |
| ET - Elution tubes, 1.5ml | 1x 25 | 1x 50 |
| Kit 1 - Manual | | |
| Manual | 1x | 1x |
| Short Manual | 3x | 3x |
| Kit 2 – Enzymes & Reagents (store at -15 to -25°C), in white box | | |
| MolDNase B, solution | 1x 0.25ml | 1x 0.5ml |
| BugLysis, solution | 1x 0.5ml | 1x 1.0ml |
| β -mercaptoethanol, solution | 1x 0.08ml | 1x 0.08ml |
| Proteinase K, solution | 1x 1.0ml | 2x 1.0ml |
| Kit 3 – Consumables (store at +18 to +25°C), in green box | | |
| 50ml Centrifuge tubes | 1x 25 | 2x 25 |

Symbols

Symbols used in labelling and in section 'Hazard and Precautionary Statements' (see pages 6 to 7).

| | | | | | |
|---|-----------------------------------|---|------------------------------|---|---------------------------|
|  | Content of the package |  | Catalogue number |  | Irritant |
|  | Manufactured by |  | Consult instructions for use |  | Health hazard |
|  | Use by |  | Flammable |  | Environmentally Hazardous |
|  | Temperature limitation (store at) |  | Corrosive | | |
|  | Batch code |  | Toxicity | | |

Storage and Stability

Guarantee for full performance of *Ultra-Deep Microbiome Prep10* 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).

50 ml Centrifuge tubes (Kit 3) are provided with the kit and have to be stored at +18 to +25°C.

Stable for 24 months from the date of manufacturing under proper storage condition. 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 Prep10 is intended as a kit for **research-use-only** and **must not be used for in-vitro diagnostic use**.

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 Prep10](#).

Sample Preparation:

- 1x thermomixer (24x 2.0ml tubes), e.g., Eppendorf comfort, Eppendorf, Germany
- 1x vortexer, e.g., VWR, Germany
- 1x bench top microcentrifuge ($\geq 12,000\times g$), e.g., miniSpin, Eppendorf, Germany
- 1x high speed centrifuge and fixed angle rotor for *50ml Centrifuge tubes* (9,500xg)
- 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 1.5ml 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 10µl, 20µl, 100µl, 200µl and 1000µl, 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
 - 10µl type Eppendorf (70.1115.210)
 - 100µl type Eppendorf (70.760.212)
 - 300µl type Eppendorf (70.765.210)
 - 1000µl type Eppendorf (70.762.211)
- 1.5 ml micro tubes, Biosphere®, Sarstedt, Germany (72.706.200)
- 10ml disposable pipette, sterile, single packed, Greiner bio-one, Germany (607180)
- 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 spilled, clean with suitable laboratory detergent and water. If the spilled 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 7)}:
H302-H315-H319; P301+P312-P302+P352-P305+P351+P338

Proteinase *K*

Contains *Proteinase K* (≥1%):
Respiratory sensitization, irritating (eyes and skin) and specific target organ toxicity – single exposure



Danger

Hazard and precautionary statements^{*(page 7)}:
H315-H319-H334-H335; P302+P352-P304+P341-P305+P351+P338

Buffer *CS*

Contains guanidinium thiocyanate (>10%):
Acute toxicity (oral, dermal, inhalation) and chronic aquatic toxicity.



Warning

Hazard and precautionary statements^{*(page 7)}:
H302-H312-H332-H412-EUH032; P260-P273-P301+P312-P302+P350-P304+P340

Buffer AB

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



Danger

Hazard and precautionary statements*:

H225-H319-H336; P210-P233-P304+P340-P305+P351+P338

Buffer WB

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



Danger

Hazard and precautionary statements*:

H225-H319-H336; P210-P233-P304+P340-P305+P351+P338

70% Ethanol, DNA-free

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



Danger

Hazard and precautionary statements*:

H225; P210-P233

β-mercaptoethanol

Contains 2-mercaptoethanol (100%): **Poisonous, irritating, environmental hazardous**



Danger

Hazard and precautionary statements*:

H227-H301-H310+H330-H315-H318-H410; P273-P301+P310-P302+P352-P304+P340-P305+P351+P338

Emergency Information (24-hours service)

Emergency medical information in English, French, and German can be obtained

24 hours a day from: Poison Information Centre Mainz, Germany; Tel: +49(0)6131 19240

Outside of Germany: Please contact the regional company representation in your country.

* **H225:** Highly flammable liquid and vapour; **H227:** Combustible liquids; **H301:** Toxic if swallowed; **H302:** Harmful if swallowed; **H302+312+332:** Harmful if swallowed, in contact with skin or if inhaled; **H310+H330:** Fatal if swallowed or in contact with skin; **H311+H331:** Toxic in contact with skin or if inhaled; **H312:** Harmful in contact with skin; **H315:** Causes skin irritation; **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; **H335:** May cause respiratory irritation; **H336:** May cause drowsiness or dizziness; **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/sparks/open flames/hot surfaces. – No-smoking; **P233:** Keep container tightly closed; **P260:** Do not breathe fume/gas/mist/vapours; **P273:** Avoid release to the environment; **P301+P310:** IF SWALLOWED: Immediately call a POISON CENTER or doctor/physician; **P301+P312:** IF SWALLOWED: Rinse mouth. Do NOT induce vomiting; **P302+P350:** IF ON SKIN: Gently wash with plenty of soap and water; **P302+P352:** IF ON SKIN: Wash with plenty of soap and water; **P304+P340:** IF INHALED: Remove to fresh air and keep at rest in a position comfortable for breathing; **P304+P341:** IF INHALED: If breathing difficult, remove to fresh air and keep at rest in a position comfortable for breathing; **P305+P351+P338:** IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

Kit Description

The *Ultra-Deep Microbiome Prep10* Procedure

In its concept, *Ultra-Deep Microbiome Prep10* is a pre-analytical means of molecular analysis of microbial communities from a variety of body sites.

Ultra-Deep Microbiome Prep10 combines new solutions for DNA extraction from 1ml to 10ml fluid samples (e.g., ascites, BAL, CSF, synovial fluid) and tissue samples (e.g., abscesses, biopsies, native/prosthetic heart valves). The procedure includes the removal of host DNA ($\geq 95\%$) and the extraction of DNA from bacteria and fungi from clinical specimens for Next-Generation Sequencing 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).

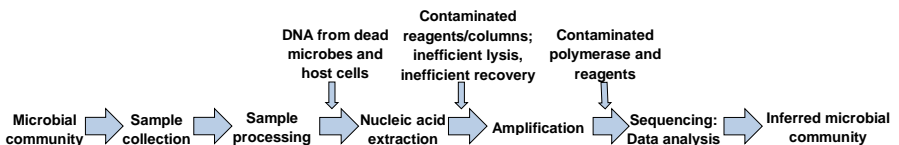


Fig. 1: Steps in the processing of samples for Next-Generation Sequencing analysis (adapted from Rogers & Bruce, 2010) and pre-analytic parameters negatively influencing the results. Thereby, the inferred microbial community structure and function may be significantly divergent from the real microbial 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 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 Prep10* contributes by supplying innovative solutions.

The analysis of specimens using *Ultra-Deep Microbiome Prep10* follows a sequence of steps (see Fig. 2). 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.

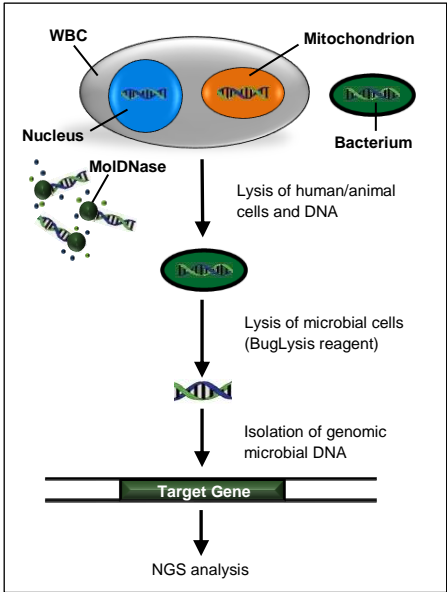


Fig. 2: The principle of enrichment and extraction of microbial DNA from specimens by *Ultra-Deep Microbiome Prep10*.

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.

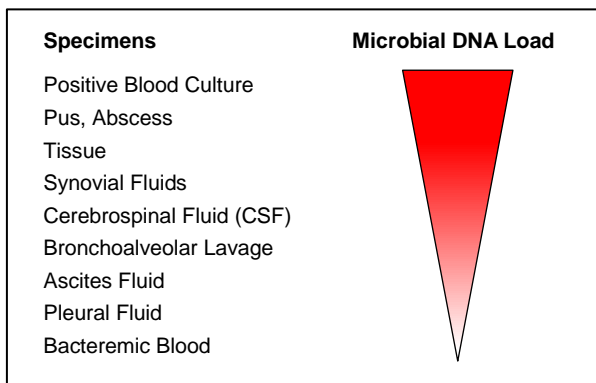


Fig. 3: Microbial DNA loads of a variety of specimens. Full-grown blood cultures can contain $>10^9$ cfu/ml, while bacteremic blood usually contains <10 - 100 cfu/ml.

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 1000cfu/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 is measured by an internal control assay.

Validation

Broad-range lysis of microorganisms

The broad-range lysis capacity of *Ultra-Deep Microbiome Prep10* 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: Microorganisms identified in various clinical specimens.

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

- ! Use only provided *50ml Centrifuge tubes* (Kit 3) in order to guarantee full performance. Do not exceed 9.500xg as maximum speed for centrifugation.
- ! 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 1.5ml 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 1ml to 10ml (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 10ml volume

Samples less than 10ml (1 to <10ml) are filled up using buffer *SU*. Use a sterile, disposable 10ml pipette and transfer the sample into a supplied sterile *50ml Centrifuge tube* (Kit 3). Then add buffer *SU* using a disposable 10ml pipette until reaching the 10ml mark of the tube. Discard pipette/pipette tip with excess buffer *SU*. Never re-use any pipette after use. Continue with part B (below).

B) Sample pre-treatment and DNA isolation procedure

1. Use a sterile, disposable 10ml pipette and transfer 10ml sample into a supplied sterile *50ml Centrifuge tube* (Kit 3) or use filled-up sample (part A, above). Then add 4ml buffer *CM* to the sample. Vortex at full speed for 15s. Let stand at room temperature (+18 to +25°C) for 5min.

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 4ml buffer *DB1* and 10µl *MolDNase B* (do not premix) to the lysate and immediately vortex for 15s. Let stand at room temperature (+18 to +25°C) for 15min.

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 high speed centrifuge at 9,500xg for 10min. Thereafter, carefully decant the supernatant.

Human cell debris and potentially present microbial cells are sedimented.

4. Pipette 1ml buffer *RS* to the sediment and resuspend by pipetting.

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. Transfer the suspension by pipetting to a 2ml *Sample tube* (*ST tube*, Kit 1). Centrifuge the *ST tube* in a bench top microcentrifuge ($\geq 12,000xg$) for 5min. 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 80µl 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 20µl *BugLysis* and 1.4µl *β-mercaptoethanol* (Kit 2) directly into the extract. Vortex the tube for 15s and incubate in a thermomixer at 37°C and 1,000rpm for 30min.

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

Caution: β-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 150µl buffer *RP* and 20µl *Proteinase K* to the *ST tube*. Vortex at full speed for 15s and incubate at 56°C and 1,000rpm for 10min.
9. Briefly centrifuge the *ST tube* and pipette 250µl buffer *CS* into it. Vortex tube at full speed for 15s.

Cells are lysed and protein is denatured.

10. Briefly centrifuge and add 250µl binding buffer *AB* to the *ST tube*. Vortex at full speed for 15s.
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 $\geq 12,000\times g$ for 30s (or minimum time of the centrifuge, e.g. 60s).

At this point nucleic acids bind to the matrix.

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

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 1.5ml *Elution tube* (*ET*; Kit 1).
15. Pipette 100µl 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 1min. Thereafter, centrifuge at $\geq 12,000\times g$ 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 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 180µl 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 0.5cm. 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 20µl of *Proteinase K* (Kit 2) to the specimen.
- Vortex the *ST tube* at full speed for 15s and incubate in the thermomixer at 56°C and 1,000rpm for 10min.

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

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 250µl buffer *DB1* to the *ST tube*. Thereafter, pipette 10µl *MolDNase B* to the lysate in the *ST tube*. Immediately vortex for 15s and let stand for 15min at room temperature (+18 to +25°C).

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 $\geq 12,000xg$ for 10min. Thereafter, carefully remove the supernatant by pipetting and discard.

Debris from lysed tissue and potentially present microbial cells are sedimented.

4. Pipette 1ml 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 $\geq 12,000xg$ for 5min. 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 80µl 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 20µl *BugLysis* and 1.4µl *β-mercaptoethanol* (Kit 2) directly into the extract. Vortex the tube for 15s and incubate in a thermomixer at 37°C and 1,000rpm for 30min.

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

Caution: β-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 150µl buffer *RP* and 20µl *Proteinase K* to the *ST tube*. Vortex at full speed for 15s and incubate at 56°C and 1,000rpm for 10min.
9. Briefly centrifuge the *ST tube* and pipette 250µl buffer *CS* into it. Vortex tube at full speed for 15s.

Microbial cells are lysed and protein is denatured.

10. Briefly centrifuge and add 250µl binding buffer *AB* to the *ST tube*. Vortex at full speed for 15s.
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 $\geq 12,000 \times g$ for 30s (or minimum time of the centrifuge, e.g. 60s).

At this point nucleic acids bind to the matrix.

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

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 1.5ml *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 $\geq 12,000 \times g$ 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 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:

Phone: +49(0)421 69 61 62 0 • **E-mail:** support@molzym.com

| Observation | Possible cause | Comments/suggestions |
|--|---|---|
| Strong human/animal DNA background in gel electrophoresis | <ul style="list-style-type: none">• Buffer <i>CM</i> not added• Buffer <i>DB1</i> not added• <i>MolDNase B</i> 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 PCR reaction. Ensure that buffer <i>CM</i> has been added to lyse human cells. Accordingly, addition of buffer <i>DB1</i> and <i>MolDNase B</i> is obligate. Keep the <i>MolDNase B</i> 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) | <ul style="list-style-type: none">• PCR inhibitors co-eluted | Check whether <i>Proteinase K</i> treatment has been performed during DNA preparation. Make sure that all washing steps of the procedure have been followed. Optionally, after 70% <i>Ethanol</i> washing (step 14 of protocols 1 and. 2), centrifuge for another 1min to avoid ethanol carry over to the eluate. |
| False positive result (signal in PCR negative control) | <ul style="list-style-type: none">• 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 | <ul style="list-style-type: none"> <li data-bbox="376 204 555 228">• Insufficient lysis <li data-bbox="376 256 555 280">• PCR inhibition <li data-bbox="376 459 555 507">• Insufficient homogenization <li data-bbox="376 536 555 584">• Microbe number too low <li data-bbox="376 687 555 759">• Loss of nucleic acids during purification <li data-bbox="376 799 555 871">• Suboptimal elution conditions <li data-bbox="376 935 555 1031">• Loss of nucleic acids during the storage of the eluate | <p data-bbox="575 204 983 427">Make sure that <i>BugLysis</i> has been added. Ensure that the <i>Proteinase K</i> treatment has been performed. Run the internal control assay (<i>Ultra-Deep Microbiome Ampli IC</i>, order no. G-130-042) for testing for potential PCR inhibition. Check whether the amplification conditions are optimal with regard to primer annealing, reaction times and cycle numbers.</p> <p data-bbox="575 459 983 632">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).</p> <p data-bbox="575 687 983 759">Ensure that buffer <i>AB</i> has been added to and mixed with the lysate (step 10 of protocols 1 and 2).</p> <p data-bbox="575 799 983 871">Make sure to elute with heated DNA-free water (70°C; step 15 of protocols 1 and 2). This increases the DNA yield significantly.</p> <p data-bbox="575 935 983 1078">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).</p> |

Tradenames

Ultra-Deep Microbiome Prep10 (Molzym), Biosphere® (Sarstedt), BioBall® MultiShot 550 KBE (bioMérieux).

References

- Arrazuria R, Elguezabal N, Juste RA, Derakhshani H, Khafipour E** (2016) Mycobacterium avium subspecies paratuberculosis infection modifies gut microbiota under different dietary conditions in a rabbit model. *Front Microbiol* 7, 446. doi: 10.3389/fmicb.2016.00446.
- Bergman M, Sune D, Nilsson A, Serrander L** (2016): Improved sensitivity by optimizing the DNA extraction and PCR in molecular detection (16S) of bacteria from tissue: ePoster EV0516, ECCMID 2016
- Espy M.J., Uhl J.R., Sloan L.M., Buckwalter S.P., Jones M.F., Vetter E.A., Yao J.D.C., Wengenack N.L., Rosenblatt J.E., Cockerill F.R. and Smith T.F.** (2006) Real-Time PCR in clinical microbiology: applications for routine laboratory testing. *Clin. Microbiol. Rev.* 19: 165-256
- Gebert S., Siegel D., Wellinghausen N.** (2008) Rapid detection of pathogens in blood culture bottles by real-time PCR in conjunction with the pre-analytic tool MolYsis. *J. Infect.*: doi:10.1016/j.jinf.2008.07.013.
- Handschr M., Karlic H., Hertel C., Pfeilstöcker M., Haslberger A.G.** (2008) Pre-analytic removal of human DNA eliminates false signals in general 16S rDNA PCR monitoring of bacterial pathogens in blood. *Comp. Immun. Microbiol. Infect. Dis.*: doi:10.1016/j.cimid.2007.10.005.
- Horz H.-P., Scheer S., Huenger, F., Vianna M., Conrads G.** (2007) Selective isolation of bacterial DNA from human clinical specimens. *J. Microbiol. Meth.* 72: 98-102.
- Liste der vom Robert-Koch-Institut** geprüften und anerkannten Desinfektionsmittel und -verfahren (2007) *Bundesgesundheitsblatt - Gesundheitsforschung - Gesundheitsschutz* 50:1335–1356
- Richtlinie für Krankenhaushygiene** und Infektionsprävention (31.11.1996). Robert Koch Institut
- Richtlinie über die ordnungsgemäße Entsorgung** von Abfällen aus Einrichtungen des Gesundheitsdienstes vom 01.01.2002, Robert-Koch-Institut
- Rogers G.B., Bruce K.D.** (2010) Next-generation sequencing in the analysis of human microbiota. *Mol. Diagn. Ther.* 14: 343-350
- Roth A., Mauch H. and Göbel U.B.** (2001) Quality standards for microbiological diagnostic techniques for infectious diseases - 1. Nucleic acid amplification techniques. Urban & Fischer Verlag, München-Jena
- Ruppé E, Greub G, Schrenzel J** (2017) Messages from the first International Conference on Clinical Metagenomics (ICCMg). *Microbes Inf*, doi.org/10.1016/j.micinf.2017.01.005.
- Ruppe E, Lazarevic V, Girard M, Mouton W, Ferry T, Laurent F, Schrenzel J** (2017) Clinical metagenomics of bone and joint infections: a proof of concept study: bioRxiv 090530; doi: <https://doi.org/10.1101/090530>
- Wellinghausen N., Kochem A.J., Disqué C., Mühl H., Gebert S., Winter J., Matten J. and Sakka S.G.** (2009) Diagnosis of bacteremia in whole-blood samples by use of a commercial universal 16S rRNA gene-based PCR and sequence analysis. *J. Clin. Microbiol.* 47: 2759-2765

Associated Products

A common drawback of PCR assays targeting bacterial sequences is the contamination of amplification reagents by bacterial DNA. This problem becomes even more evident when the assay is directed to a broad range of bacteria. 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 | 100 reactions | S-020-0100 |
| Universal 16S rDNA PCR and Real-Time | 250 reactions | S-020-0250 |
| PCR assay for detection of bacterial DNA | 1000 reactions | S-020-1000 |
| Mastermix 16S Primer | 100 reactions | S-021-0100 |
| PCR assay for universal PCR detection of | 250 reactions | S-021-0250 |
| bacterial DNA | 1000 reactions | S-021-1000 |
| Mastermix 18S Complete | 100 reactions | S-070-0100 |
| Universal 18S rDNA PCR and Real-Time | 250 reactions | S-070-0250 |
| PCR assay for detection of fungal DNA | 1000 reactions | S-070-1000 |
| Mastermix 16S/18S Dye | 100 reactions | S-030-0100 |
| Premixed reagents and fluorescent dye for | 250 reactions | S-030-0250 |
| Real-Time PCR with custom primers | 1000 reactions | S-030-1000 |
| Mastermix 16S/18S Basic | 100 reactions | S-040-0100 |
| Premixed reagents for PCR analysis with | 250 reactions | S-040-0250 |
| custom primers | 1000 reactions | S-040-1000 |
| Taq DNA Polymerase, DNA-free | | |
| MolTaq 16S/18S | 100 units | P-019-0100 |
| | 500 units | P-019-0500 |
| Hot MolTaq 16S/18S | 100 units | P-080-0100 |
| | 500 units | P-080-0500 |
| PCR-Grade Water, DNA-free | | |
| DNA-free water, PCR grade | 10x 1.7ml | P-020-0003 |

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. |
|-------------------------------------|-----------------------|-----------|
| <i>Ultra-Deep Microbiome Prep</i> | | |
| 0.2-1ml fluid samples & | 25 sample extractions | G-020-025 |
| 0.5x0.5cm tissue samples | 50 sample extractions | G-020-050 |
| <i>Ultra-Deep Microbiome Prep10</i> | | |
| 1-10ml fluid samples & | 25 sample extractions | G-030-025 |
| 0.5x0.5cm tissue samples | 50 sample extractions | G-030-050 |

Order Hotline:

Tel.: +49(0)421 69 61 62 0 • **Fax:** +49(0)421 69 61 62 11 • **E-Mail:** order@molzym.com

Contact

Molzym GmbH & Co KG

Mary-Astell Str. 10
28359 Bremen, Germany

Tel.: +49(0)421 69 61 62 0 • **Fax:** +49(0)421 69 61 62 11

E-Mail: info@molzym.com • **Web:** www.molzym.com