Umwelt Proben Bank
Des Bundes

Guidelines for Sampling and Sample Processing

# Storage of Environmental Samples under Cryogenic Conditions

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## Contents

# 1 German Environmental Specimen Bank

The German Environmental Specimen Bank (ESB) is an instrument for the monitoring of the environment. It is in the responsibility of the Federal Ministry for the Environment, Nature Protection and Reactor Safety (BMU) and technically and administratively coordinated by the Federal Environment Agency (Umweltbundesamt). The ESB collects ecologically representative environmental specimens as well as human samples, stores them and examines the archived material for environmental relevant substances.

The long-term storage is performed under conditions that exclude a change of state or a loss of chemical characteristics as far as possible during a period of several decades. By this means the archive provides specimens for a retrospective monitoring of such substances, whose hazard potential for the environment or human health are not yet known.

Comprehensive information on the German ESB is available at www.umweltprobenbank.de (English language pages available).

# 2 General information

This protocol applies to biological environmental samples like bladder wrack, bream muscle, earthworm, spruce shoots as well as abiotic samples such as soil and suspended particulate matter.

For the storage in the ESB archive all samples are frozen immediately at the time of the sampling and the cooling chain is not interrupted at any time afterwards. Thus transport, storage as well as grinding and homogenization of the sample material takes place under cryogenic conditions (temperature below approx. -130°C) considering special safety regulations.

The particularly high quality assurance requirements result from the extraordinary importance of the samples as archive material. Representativeness and reproducibility of the specimens are prerequisites for the comparability of the analytical data in time and space.

The aim of this guideline is to provide information on the safe storage of sensitive sample material under cryogenic conditions in compliance with safety regulations and without deterioration of sample quality or loss of information on the substances contained in the samples.

# 3 Terminology

## 3.1 Cryogenic conditions

Cryogenic conditions exist at temperatures of below approx. -130°C (approx. < 140 K). The temperature is therefore below the glass transition temperature of water. No further re-crystallisation of ice takes place and no further ice crystals form (BURDEN 1999). This ensures that chemical processes in the samples are reduced to a minimum and morphological changes, e.g. through further growth of ice crystals, are prevented. During storage in the gas phase over liquid nitrogen, an inert-gas atmosphere is created by the nitrogen evaporating in the sample-storage container. This also prevents any changes in the samples caused by oxygen from the atmosphere (oxidation processes).

# 4 Safety notes for working with liquid nitrogen

Caution: Liquid nitrogen is extremely cold (i.e. skin and tissue may be damaged on contact) and may cause asphyxiation when evaporating. When liquid nitrogen is being used in enclosed rooms, sufficient ventilation is essential. The oxygen content of the room atmosphere should be monitored with a portable oxygen sensor or a stationary oxygen-deficiency alarm system (settings: e.g. first alarm at oxygen content $\leq$ 19 %, and main alarm at $\leq$ 17 %).

Always follow the instructions for use of liquid nitrogen (appendix A).

## 5 Storage operation

### 5.1 Building

For storage under cryogenic conditions, a room or building is required which is fitted with a controlled ventilation system (e.g. three or four changes of air per hour, filtered air supply) to prevent accumulation of evaporating nitrogen in the atmosphere. The oxygen content must also be continuously monitored. Unauthorised persons should have no access to the building.

Samples are stored in a separate building. Under normal conditions, a complete exchange of air takes place three or four times per hour. In emergency operation (i.e. when the oxygen content in the atmosphere falls below 17%) the air is exchanged seven or eight times per hour. The air supply is cleaned by means of particle and activated-carbon filters. Sensors are installed for continuous monitoring of atmospheric oxygen in both buildings. Access to the storage buildings is controlled electronically. Access is allowed only to authorised persons.

### 5.2 System description

The system used for storing samples consists of the following components:

1. Cryogenic storage containers with liquid-nitrogen cooling: containers from various manufacturers (e.g. Chronos 1400 from Cryotherm, or ABC 1400-B from Achenbach Buschhütten Cryotechnik, each with approx. 1400 litres capacity).

2. Equipment for monitoring the level and controlling the automatic filling of the cryogenic storage containers with liquid nitrogen (ADUR equipment from Cryotherm).

3. Equipment for controlling the alarm parameters (e.g. temperature or low nitrogen level) and giving alarm when necessary via a text message system or telephone dialling unit. The Biosafe system produced by Cryotherm is used. This is a computer-assisted monitoring system using the Cryotherm Biosafe software. It has two groups of programs each of which monitors up to 32 cryogenic containers (Biosafe 1 and Biosafe 2 for up to 64 containers in all). The system is connected to the general control and monitoring system of the institute for sending alarm calls to the mobile telephones used by the standby service (types of alarm given: e.g. low nitrogen, overfilling, temperature exceeded). Other disturbances are also detected and alarm signals given to the standby service (e.g. power cut, computer failure, low oxygen level).

4. Vacuum-insulated pipes for supplying the cryogenic containers with liquid nitrogen (from Cryotherm).

5. Depending on the system, a phase separator may also be used to separate the gaseous nitrogen in order to keep the vacuum-insulated pipes completely filled with liquid nitrogen at all times (from Cryotherm).

6. Storage tank for liquid nitrogen (rented tank from Air Liquide with a capacity of approx. 14000 litres sufficient for at least 10 days. The tank is refilled at weekly intervals).

7. Sample-storage system, preferably of metal: aluminium canisters and storage frames specially designed for scintillation vials (diameter approx. 26 mm, height approx. 58 mm, volume approx 20 ml), or for Duran-glass flasks with screw-on caps. Raw sample material is stored in stainless-steel containers (square type with standardised volumes of 1.5 litres, 3.5 litres and 5.5 litres as used in gastronomy) which are then stored in the cryogenic storage containers (for easier removal generally in 1000 litre containers).

8. Liquid nitrogen of quality 5.0 ($\geq$ 99.999 %; e.g. supplied by Air Liquide, product no. 0319, see data sheet Appendix B).

## 6 Standard process

### 6.1 Safety regulations

Always comply with the instructions for working with liquid nitrogen (see Appendix A). The oxygen content of the room atmosphere must be monitored with either a fixed system or with portable oxygen sensors. Always ensure that the storage room is adequately ventilated. Always wear protective clothing (goggles, lab coat, insulated safety gloves) when working with liquid nitrogen.

### 6.2 Storage conditions

All samples are stored over liquid nitrogen in the gas phase. According to the manufacturer's data, the evaporation rate is approximately 1% of the container volume of liquid nitrogen per day with the container closed (i.e. approx. 14 litres per 1400-litre container) or approx. 20 litres of liquid nitrogen per day with an average amount of handling (occasional removal of samples from or placing of samples in the cryo-storage container). When the minimum level is reached, the solenoid valve opens automatically, and the container is topped up with liquid nitrogen from the phase separator.

### 6.3 Automatic filling control and alarm system

'Automatic filling' should be set permanently on the ADUR units to ensure that the cryo-storage containers are kept filled at all times.
The covers of the units must be kept closed to avoid accidental re-setting to manual filling. The level indicator of the ADUR units indicates the level of liquid nitrogen by means of 5 LEDs. 'Alarm overfill', 'Maximum', 'Fill', 'Minimum', 'Alarm underfill'. When functioning normally, liquid nitrogen is refilled automatically when the level drops below 'Minimum'. Manual refilling is also possible.

### 6.4 Checks

The entire storage system must be checked once per working day (visual check of cryo-storage containers, check of monitoring computer). The storage tank must be refilled regularly (normally once per week). Before public holidays it is particularly important to ensure that sufficient liquid nitrogen is available. If necessary, an additional delivery should be ordered at short notice. An appropriate agreement should be made with the supplier.

If a computer system is being used to monitor and record alarms, data should be saved at regular intervals. In the case of the Biosafe this operation must be carried out separately for each of the two program groups (Biosafe 1 and Biosafe 2).

### 6.5 Cleaning

Should cleaning be required, the cryogenic storage containers should be allowed to warm up to room temperature. The surface should then be washed with a mild cleaning agent and allowed to dry completely. The cover should not be replaced until the container is dry. The cleaning procedure is important in order to avoid contamination of the samples and possible health hazard through microbial contamination.

### 6.6 Maintenance

It is recommended to inspect the entire liquid nitrogen system once per year by a specialist company. The function of all components should be checked. This includes, for example, checking and adjusting the sensors for detecting the level of liquid nitrogen in the cryo-storage containers and a test of the alarm function.

### 6.7 Emergency precautions

Suitable precautions must be taken for dealing with emergencies.

It must be ensured that a backup storage container is available in case of failure of one of the cry-storage containers in use.

In case of a power cut lasting several hours, the cryo-storage containers must be filled manually (if necessary by the standby staff outside normal working hours).

If insufficient liquid nitrogen is available (e.g. through leakage of the storage tank or incorrect operation) liquid nitrogen must be supplied at short notice. An appropriate arrangement should be made with the supplier.

## 7 Organisation of standby service in case of problems

Should any problem occur in the operation of the liquid nitrogen system for supplying the cryo-storage containers, an alarm signal is given to alert the staff on standby. Trained and experienced staff (at least six persons) should be assigned to standby duties in advance.

These persons are trained in the use of the liquid nitrogen system and are therefore able to deal directly with most types of problem. They are alerted by the internal alarm system which sends

SMS messages to the mobile telephones of the standby personnel. The alarm must be acknowledged. With certain types of disturbances, the person on standby must inspect the system personally and take remedial action. If necessary, a second person should also be in attendance for safety reasons (when handling liquid nitrogen).

In case of problems which cannot be corrected by the staff, the emergency service of the company responsible for maintenance of the liquid nitrogen system should be alerted.

# 8 Documentation

All unusual events must be recorded and reported to the person responsible for the storage of samples. The samples in storage must also be documented in regular intervals.

All important events are entered in a storage log book. The alarms of the cryo-storage containers are also saved electronically (via the Biosafe system and software).

The specimen stock is documented in a Microsoft Access data base, by means of which stock reports can also be displayed. A report on the inventory is compiled once per year.

# 9 References

BMU (Bundesministerium für Umwelt, Naturschutz und Reaktorsicherheit, Hrsg.) (2008): German Environmental Specimen Bank – Concept (Status: October 2008); www.umweltprobenbank.de

Burden, D. W. (1999): Issues in Contamination and Temperature Variation in the Cryopreservation of Animal Cells and Tissues. Revco Technologies, Asheville, NC 28806 (USA), Application Note 99-08.

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## Appendix A: Instructions for working with liquid nitrogen

DANGER FOR PERSONS AND ENVIRONMENT

On contact with eyes or skin, cryogenic, liquid nitrogen causes serious frostbite with injuries similar to burns (i.e. inflammation, swelling, blistering) as well as severe damage to tissue. The cold gas is heavier than air, accumulates at floor level and may displace the oxygen in low-lying rooms. One litre of liquid nitrogen produces approximately 650 litres of gas! At concentrations of more than 85% in the air, severe oxygen deficiency occurs causing symptoms such as drowsiness, nausea, increase in blood pressure and difficulty in breathing. Concentrations of 88% and more, lead to immediate loss of consciousness and risk of asphyxiation.

SAFETY MEASURES AND PROCEDURES

Liquid nitrogen should be transported and handled only in suitable cryogenic vessels or apparatus which is resistant to cold. Never handle larger quantities in small poorly ventilated rooms. Always avoid contact between the cryogenic liquid or gas and the skin and eyes. Always wear protective clothing, impermeable shoes, leather safety gloves and goggles. If release of large quantities of gas is anticipated or unavoidable, also use insulating equipment. When working in confined or inadequately ventilated rooms, a second person is required for supervision outside the danger zone who can raise the alarm if necessary.

EMERGENCY PROCEDURE

Fire: Nitrogen is not inflammable. Take measures appropriate to the surroundings. If a fire cannot be extinguished immediately, leave the area immediately. Never attempt to extinguish flames with liquid nitrogen. On release of larger quantities of nitrogen, warn all other persons, leave the danger zone and re-enter it only with insulating equipment. If possible, repair the leakage. Do not re-enter the danger zone without isolating equipment and before it has been thoroughly ventilated. If necessary, measure the concentration of nitrogen in the air.

FIRST AID

Aspiration: Take affected person out of doors immediately. If necessary assist respiration with mask and bag to provide ventilation. Seek medical assistance! Eye contact: do not move or rub parts of the body which are frozen rigid. Thaw carefully with (cold) water. Remove clothing and cover the body loosely with sterile bandaging material. Seek medical assistance!

DISPOSAL

Allow to evaporate slowly in the open air. Loosely cover the opening of the vessel to avoid condensation and concentration of atmospheric oxygen in the remaining liquid.

## Appendix B: Product data sheet for liquid nitrogen

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| **Product data sheet** | **Nitrogen** | **Air Liquide Product no. 0319** |
| Technical gases | liquid, high purity | |

Purity in vol. %: ≥ 99.999%

Other gases:

$O_2$ ≤ 2 ppm (v/v)

$H_2O$ ≤ 1 ppm (v/v)

Safety information: EU Safety Data Sheet according to TRGS 220 available

Conversion factors:

| | $m^3$ Gas (1 bar, 15°C) | Litre liquid (boiling temp., at 1 bar) | kg |
|---|---|---|---|
| $m^3$ | 1 | 1.448 | 1.170 |
| Litre | 0.691 | 1 | 0.808 |
| kg | 0.855 | 0.238 | 1 |

Formula: $N_2$

Main characteristics:

inert, non-inflammable,
non-toxic, asphyxiant in high concentrations,
deep-cold liquid gas may cause frostbite,
concentration in ambient atmosphere 78,08 vol.%.

Physical and chemical properties

| | |
|---|---|
| Appearance: | colourless |
| Odour: | odourless |
| Mol mass: | 28.01 g/mol |
| State at 20°C: | gaseous |
| Triple point: | -210°C (at 1.013 bar) |
| Boiling point: (sublimation) | -196°C (at 1.013 bar) |
| Critical temperature: | -147°C |
| Critical pressure: | 34 bar |
| Density, liquid (boiling temp. 1 bar): | 812 kg/$m^3$ |
| Density, gaseous (15°C, 1 bar): | 1.17 kg/$m^3$ |
| Relative density, gaseous (air = 1): | 0.97 |
| Solubility in water (20°C, 1 bar): | 20 mg/L |