
Security and resilience — Business continuity management systems — Requirements

*Sécurité et résilience — Systèmes de management de la continuité
d'activité — Exigences*





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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 292, *Security and resilience*.

This second edition cancels and replaces the first edition (ISO 22301:2012), which has been technically revised. The main changes compared with the previous edition are as follows:

- ISO's requirements for management system standards, which have evolved since 2012, have been applied;
- requirements have been clarified, with no new requirements added;
- discipline-specific business continuity requirements are now almost entirely within [Clause 8](#);
- [Clause 8](#) has been re-structured to provide a clearer understanding of the key requirements;
- a number of discipline-specific business continuity terms have been modified to improve clarity and to reflect current thinking.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

0.1 General

This document specifies the structure and requirements for implementing and maintaining a business continuity management system (BCMS) that develops business continuity appropriate to the amount and type of impact that the organization may or may not accept following a disruption.

The outcomes of maintaining a BCMS are shaped by the organization's legal, regulatory, organizational and industry requirements, products and services provided, processes employed, size and structure of the organization, and the requirements of its interested parties.

A BCMS emphasizes the importance of:

- understanding the organization's needs and the necessity for establishing business continuity policies and objectives;
- operating and maintaining processes, capabilities and response structures for ensuring the organization will survive disruptions;
- monitoring and reviewing the performance and effectiveness of the BCMS;
- continual improvement based on qualitative and quantitative measures.

A BCMS, like any other management system, includes the following components:

- a) a policy;
- b) competent people with defined responsibilities;
- c) management processes relating to:
 - 1) policy;
 - 2) planning;
 - 3) implementation and operation;
 - 4) performance assessment;
 - 5) management review;
 - 6) continual improvement;
- d) documented information supporting operational control and enabling performance evaluation.

0.2 Benefits of a business continuity management system

The purpose of a BCMS is to prepare for, provide and maintain controls and capabilities for managing an organization's overall ability to continue to operate during disruptions. In achieving this, the organization is:

- a) from a business perspective:
 - 1) supporting its strategic objectives;
 - 2) creating a competitive advantage;
 - 3) protecting and enhancing its reputation and credibility;

- 4) contributing to organizational resilience;
- b) from a financial perspective:
 - 1) reducing legal and financial exposure;
 - 2) reducing direct and indirect costs of disruptions;
- c) from the perspective of interested parties:
 - 1) protecting life, property and the environment;
 - 2) considering the expectations of interested parties;
 - 3) providing confidence in the organization's ability to succeed;
- d) from an internal processes perspective:
 - 1) improving its capability to remain effective during disruptions;
 - 2) demonstrating proactive control of risks effectively and efficiently;
 - 3) addressing operational vulnerabilities.

0.3 Plan-Do-Check-Act (PDCA) cycle

This document applies the Plan (establish), Do (implement and operate), Check (monitor and review) and Act (maintain and improve) (PDCA) cycle to implement, maintain and continually improve the effectiveness of an organization's BCMS.

This ensures a degree of consistency with other management systems standards, such as ISO 9001, ISO 14001, ISO/IEC 20000-1, ISO/IEC 27001 and ISO 28000, thereby supporting consistent and integrated implementation and operation with related management systems.

In accordance with the PDCA cycle, [Clauses 4](#) to [10](#) cover the following components.

- [Clause 4](#) introduces the requirements necessary to establish the context of the BCMS applicable to the organization, as well as needs, requirements and scope.
- [Clause 5](#) summarizes the requirements specific to top management's role in the BCMS, and how leadership articulates its expectations to the organization via a policy statement.
- [Clause 6](#) describes the requirements for establishing strategic objectives and guiding principles for the BCMS as a whole.
- [Clause 7](#) supports BCMS operations related to establishing competence and communication on a recurring/as-needed basis with interested parties, while documenting, controlling, maintaining and retaining required documented information.
- [Clause 8](#) defines business continuity needs, determines how to address them and develops procedures to manage the organization during a disruption.
- [Clause 9](#) summarizes the requirements necessary to measure business continuity performance, BCMS conformity with this document, and to conduct management review.
- [Clause 10](#) identifies and acts on BCMS nonconformity and continual improvement through corrective action.

0.5 Contents of this document

This document conforms to ISO's requirements for management system standards. These requirements include a high level structure, identical core text and common terms with core definitions, designed to benefit users implementing multiple ISO management system standards.

This document does not include requirements specific to other management systems, though its elements can be aligned or integrated with those of other management systems.

This document contains requirements that can be used by an organization to implement a BCMS and to assess conformity. An organization that wishes to demonstrate conformity to this document can do so by:

- making a self-determination and self-declaration; or
- seeking confirmation of its conformity by parties having an interest in the organization, such as customers; or
- seeking confirmation of its self-declaration by a party external to the organization; or
- seeking certification/registration of its BCMS by an external organization.

[Clauses 1](#) to [3](#) in this document set out the scope, normative references and terms and definitions that apply to the use of this document. [Clauses 4](#) to [10](#) contain the requirements to be used to assess conformity to this document.

In this document, the following verbal forms are used:

- a) “shall” indicates a requirement;
- b) “should” indicates a recommendation;
- c) “may” indicates a permission;
- d) “can” indicates a possibility or a capability.

Information marked as “NOTE” is for guidance in understanding or clarifying the associated requirement. “Notes to entry” used in [Clause 3](#) provide additional information that supplements the terminological data and can contain provisions relating to the use of a term.

Security and resilience — Business continuity management systems — Requirements

1 Scope

This document specifies requirements to implement, maintain and improve a management system to protect against, reduce the likelihood of the occurrence of, prepare for, respond to and recover from disruptions when they arise.

The requirements specified in this document are generic and intended to be applicable to all organizations, or parts thereof, regardless of type, size and nature of the organization. The extent of application of these requirements depends on the organization's operating environment and complexity.

This document is applicable to all types and sizes of organizations that:

- a) implement, maintain and improve a BCMS;
- b) seek to ensure conformity with stated business continuity policy;
- c) need to be able to continue to deliver products and services at an acceptable predefined capacity during a disruption;
- d) seek to enhance their resilience through the effective application of the BCMS.

This document can be used to assess an organization's ability to meet its own business continuity needs and obligations.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 22300, *Security and resilience — Vocabulary*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 22300 and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <http://www.electropedia.org/>

NOTE The terms and definitions given below supersede those given in ISO 22300:2018.

3.1

activity

set of one or more tasks with a defined output

[SOURCE: ISO 22300:2018, 3.1, modified — The definition has been replaced and the example has been deleted.]

3.2

audit

systematic, independent and documented *process* (3.26) for obtaining audit evidence and evaluating it objectively to determine the extent to which the audit criteria are fulfilled

Note 1 to entry: An audit can be an internal audit (first party) or an external audit (second party or third party), and it can be a combined audit (combining two or more disciplines).

Note 2 to entry: An internal audit is conducted by the *organization* (3.21) itself, or by an external party on its behalf.

Note 3 to entry: "Audit evidence" and "audit criteria" are defined in ISO 19011.

Note 4 to entry: The fundamental elements of an audit include the determination of the *conformity* (3.7) of an object according to a procedure carried out by personnel not being responsible for the object audited.

Note 5 to entry: An internal audit can be for management review and other internal purposes and can form the basis for an organization's declaration of conformity. Independence can be demonstrated by the freedom from responsibility for the *activity* (3.1) being audited. External audits include second- and third-party audits. Second-party audits are conducted by parties having an interest in the organization, such as customers, or by other persons on their behalf. Third-party audits are conducted by external, independent auditing organizations, such as those providing certification/registration of conformity or government agencies.

Note 6 to entry: This constitutes one of the common terms and core definitions of the high level structure for ISO management system standards. The original definition has been modified by adding Notes 4 and 5 to entry.

3.3

business continuity

capability of an *organization* (3.21) to continue the delivery of *products and services* (3.27) within acceptable time frames at predefined capacity during a *disruption* (3.10)

[SOURCE: ISO 22300:2018, 3.24, modified — The definition has been replaced.]

3.4

business continuity plan

documented information (3.11) that guides an *organization* (3.21) to respond to a *disruption* (3.10) and resume, recover and restore the delivery of *products and services* (3.27) consistent with its *business continuity* (3.3) *objectives* (3.20)

[SOURCE: ISO 22300:2018, 3.27, modified — The definition has been replaced and Note 1 to entry has been deleted.]

3.5

business impact analysis

process (3.26) of analysing the *impact* (3.13) over time of a *disruption* (3.10) on the *organization* (3.21)

Note 1 to entry: The outcome is a statement and justification of *business continuity* (3.3) *requirements* (3.28).

[SOURCE: ISO 22300:2018, 3.29, modified — The definition has been replaced and Note 1 to entry has been added.]

3.6

competence

ability to apply knowledge and skills to achieve intended results

Note 1 to entry: This constitutes one of the common terms and core definitions of the high level structure for ISO management system standards.

3.7

conformity

fulfilment of a *requirement* (3.28)

Note 1 to entry: This constitutes one of the common terms and core definitions of the high level structure for ISO management system standards.

3.8**continual improvement**

recurring *activity* (3.1) to enhance *performance* (3.23)

Note 1 to entry: This constitutes one of the common terms and core definitions of the high level structure for ISO management system standards.

3.9**corrective action**

action to eliminate the cause(s) of a *nonconformity* (3.19) and to prevent recurrence

Note 1 to entry: This constitutes one of the common terms and core definitions of the high level structure for ISO management system standards.

3.10**disruption**

incident (3.14), whether anticipated or unanticipated, that causes an unplanned, negative deviation from the expected delivery of *products and services* (3.27) according to an *organization's* (3.21) *objectives* (3.20)

[SOURCE: ISO 22300:2018, 3.70, modified — The definition has been replaced.]

3.11**documented information**

information required to be controlled and maintained by an *organization* (3.21) and the medium on which it is contained

Note 1 to entry: Documented information can be in any format and media, and from any source.

Note 2 to entry: Documented information can refer to:

- the *management system* (3.16), including related *processes* (3.26);
- information created in order for the organization to operate (documentation);
- evidence of results achieved (records).

Note 3 to entry: This constitutes one of the common terms and core definitions of the high level structure for ISO management system standards.

3.12**effectiveness**

extent to which planned *activities* (3.1) are realized and planned results achieved

Note 1 to entry: This constitutes one of the common terms and core definitions of the high level structure for ISO management system standards.

3.13**impact**

outcome of a *disruption* (3.10) affecting *objectives* (3.20)

[SOURCE: ISO 22300:2018, 3.107, modified — The definition has been replaced.]

3.14**incident**

event that can be, or could lead to, a *disruption* (3.10), loss, emergency or crisis

[SOURCE: ISO 22300:2018, 3.111, modified — The definition has been replaced.]

3.15

interested party (preferred term)

stakeholder (admitted term)

person or *organization* (3.21) that can affect, be affected by, or perceive itself to be affected by a decision or *activity* (3.1)

EXAMPLE Customers, owners, personnel, providers, bankers, regulators, unions, partners or society that can include competitors or opposing pressure groups.

Note 1 to entry: A decision maker can be an interested party.

Note 2 to entry: Impacted communities and local populations are considered to be interested parties.

Note 3 to entry: This constitutes one of the common terms and core definitions of the high level structure for ISO management system standards. The original definition has been modified by adding an example and Notes 1 and 2 to entry.

3.16

management system

set of interrelated or interacting elements of an *organization* (3.21) to establish *policies* (3.24) and *objectives* (3.20) and *processes* (3.26) to achieve those objectives

Note 1 to entry: A management system can address a single discipline or several disciplines.

Note 2 to entry: The system elements include the organization's structure, roles and responsibilities, planning and operation.

Note 3 to entry: The scope of a management system can include the whole of the organization, specific and identified functions of the organization, specific and identified sections of the organization, or one or more functions across a group of organizations.

Note 4 to entry: This constitutes one of the common terms and core definitions of the high level structure for ISO management system standards.

3.17

measurement

process (3.26) to determine a value

Note 1 to entry: This constitutes one of the common terms and core definitions of the high level structure for ISO management system standards.

3.18

monitoring

determining the status of a system, a *process* (3.26) or an *activity* (3.1)

Note 1 to entry: To determine the status, there can be a need to check, supervise or critically observe.

Note 2 to entry: This constitutes one of the common terms and core definitions of the high level structure for ISO management system standards.

3.19

nonconformity

non-fulfilment of a *requirement* (3.28)

Note 1 to entry: This constitutes one of the common terms and core definitions of the high level structure for ISO management system standards.

3.20

objective

result to be achieved

Note 1 to entry: An objective can be strategic, tactical, or operational.

Note 2 to entry: Objectives can relate to different disciplines (such as financial, health and safety, and environmental goals) and can apply at different levels (such as strategic, organization-wide, project, product and process (3.26)).

Note 3 to entry: An objective can be expressed in other ways, e.g. as an intended outcome, a purpose, an operational criterion, as a *business continuity* (3.3) objective, or by the use of other words with similar meaning (e.g. aim, goal, or target).

Note 4 to entry: In the context of business continuity *management systems* (3.16), business continuity objectives are set by the *organization* (3.21), consistent with the business continuity *policy* (3.24), to achieve specific results.

Note 5 to entry: This constitutes one of the common terms and core definitions of the high level structure for ISO management system standards.

3.21

organization

person or group of people that has its own functions with responsibilities, authorities and relationships to achieve its *objectives* (3.20)

Note 1 to entry: The concept of organization includes, but is not limited to, sole-trader, company, corporation, firm, enterprise, authority, partnership, charity or institution, or part or combination thereof, whether incorporated or not, public or private.

Note 2 to entry: For organizations with more than one operating unit, a single operating unit can be defined as an organization.

Note 3 to entry: This constitutes one of the common terms and core definitions of the high level structure for ISO management system standards. The original definition has been modified by adding Note 2 to entry.

3.22

outsource

make an arrangement where an external *organization* (3.21) performs part of an organization's function or *process* (3.26)

Note 1 to entry: An external organization is outside the scope of the *management system* (3.16), although the outsourced function or process is within the scope.

Note 2 to entry: This constitutes one of the common terms and core definitions of the high level structure for ISO management system standards.

3.23

performance

measurable result

Note 1 to entry: Performance can relate either to quantitative or qualitative findings.

Note 2 to entry: Performance can relate to managing *activities* (3.1), *processes* (3.26), products (including services), systems or *organizations* (3.21).

Note 3 to entry: This constitutes one of the common terms and core definitions of the high level structure for ISO management system standards.

3.24

policy

intentions and direction of an *organization* (3.21), as formally expressed by its *top management* (3.31)

Note 1 to entry: This constitutes one of the common terms and core definitions of the high level structure for ISO management system standards.

3.25

prioritized activity

activity (3.1) to which urgency is given in order to avoid unacceptable *impacts* (3.13) to the business during a *disruption* (3.10)

[SOURCE: ISO 22300:2018, 3.176, modified — The definition has been replaced and Note 1 to entry has been deleted.]

3.26

process

set of interrelated or interacting *activities* (3.1) which transforms inputs into outputs

Note 1 to entry: This constitutes one of the common terms and core definitions of the high level structure for ISO management system standards.

3.27

product and service

output or outcome provided by an *organization* (3.21) to *interested parties* (3.15)

EXAMPLE Manufactured items, car insurance, community nursing.

[SOURCE: ISO 22300:2018, 3.181, modified — The term "product and service" has replaced "product or service" and the definition has been replaced.]

3.28

requirement

need or expectation that is stated, generally implied or obligatory

Note 1 to entry: "Generally implied" means that it is custom or common practice for the *organization* (3.21) and *interested parties* (3.15) that the need or expectation under consideration is implied.

Note 2 to entry: A specified requirement is one that is stated, e.g. in *documented information* (3.11).

Note 3 to entry: This constitutes one of the common terms and core definitions of the high level structure for ISO management system standards.

3.29

resource

all assets (including plant and equipment), people, skills, technology, premises, and supplies and information (whether electronic or not) that an *organization* (3.21) has to have available to use, when needed, in order to operate and meet its *objective* (3.20)

[SOURCE: ISO 22300:2018, 3.193, modified — The definition has been replaced.]

3.30

risk

effect of uncertainty on *objectives* (3.20)

Note 1 to entry: An effect is a deviation from the expected — positive or negative.

Note 2 to entry: Uncertainty is the state, even partial, of deficiency of information related to, understanding or knowledge of, an event, its consequence, or likelihood.

Note 3 to entry: Risk is often characterized by reference to potential "events" (as defined in ISO Guide 73) and "consequences" (as defined in ISO Guide 73), or a combination of these.

Note 4 to entry: Risk is often expressed in terms of a combination of the consequences of an event (including changes in circumstances) and the associated likelihood (as defined in ISO Guide 73) of occurrence.

Note 5 to entry: This constitutes one of the common terms and core definitions of the high level structure for ISO management system standards. The definition has been modified to add "on objectives" to be consistent with ISO 31000.

3.31**top management**

person or group of people who directs and controls an *organization* (3.21) at the highest level

Note 1 to entry: Top management has the power to delegate authority and provide *resources* (3.29) within the organization.

Note 2 to entry: If the scope of the *management system* (3.16) covers only part of an organization, then top management refers to those who direct and control that part of the organization.

Note 3 to entry: This constitutes one of the common terms and core definitions of the high level structure for ISO management system standards.

4 Context of the organization**4.1 Understanding the organization and its context**

The organization shall determine external and internal issues that are relevant to its purpose and that affect its ability to achieve the intended outcome(s) of its BCMS.

NOTE These issues will be influenced by the organization's overall objectives, its products and services and the amount and type of risk that it may or may not take.

4.2 Understanding the needs and expectations of interested parties**4.2.1 General**

When establishing its BCMS, the organization shall determine:

- a) the interested parties that are relevant to the BCMS;
- b) the relevant requirements of these interested parties.

4.2.2 Legal and regulatory requirements

The organization shall:

- a) implement and maintain a process to identify, have access to, and assess the applicable legal and regulatory requirements related to the continuity of its products and services, activities and resources;
- b) ensure that these applicable legal, regulatory and other requirements are taken into account in implementing and maintaining its BCMS;
- c) document this information and keep it up to date.

4.3 Determining the scope of the business continuity management system**4.3.1 General**

The organization shall determine the boundaries and applicability of the BCMS to establish its scope.

When determining this scope, the organization shall consider:

- a) the external and internal issues referred to in 4.1;
- b) the requirements referred to in 4.2;
- c) its mission, goals, and internal and external obligations.

The scope shall be available as documented information.

4.3.2 Scope of the business continuity management system

The organization shall:

- a) establish the parts of the organization to be included in the BCMS, taking into account its location(s), size, nature and complexity;
- b) identify products and services to be included in the BCMS.

When defining the scope, the organization shall document and explain exclusions. They shall not affect the organization's ability and responsibility to provide business continuity, as determined by the business impact analysis or risk assessment and applicable legal or regulatory requirements.

4.4 Business continuity management system

The organization shall establish, implement, maintain and continually improve a BCMS, including the processes needed and their interactions, in accordance with the requirements of this document.

5 Leadership

5.1 Leadership and commitment

Top management shall demonstrate leadership and commitment with respect to the BCMS by:

- a) ensuring that the business continuity policy and business continuity objectives are established and are compatible with the strategic direction of the organization;
- b) ensuring the integration of the BCMS requirements into the organization's business processes;
- c) ensuring that the resources needed for the BCMS are available;
- d) communicating the importance of effective business continuity and of conforming to the BCMS requirements;
- e) ensuring that the BCMS achieves its intended outcome(s);
- f) directing and supporting persons to contribute to the effectiveness of the BCMS;
- g) promoting continual improvement;
- h) supporting other relevant managerial roles to demonstrate their leadership and commitment as it applies to their areas of responsibility.

NOTE Reference to "business" in this document can be interpreted broadly to mean those activities that are core to the purposes of the organization's existence.

5.2 Policy

5.2.1 Establishing the business continuity policy

Top management shall establish a business continuity policy that:

- a) is appropriate to the purpose of the organization;
- b) provides a framework for setting business continuity objectives;
- c) includes a commitment to satisfy applicable requirements;

- d) includes a commitment to continual improvement of the BCMS.

5.2.2 Communicating the business continuity policy

The business continuity policy shall:

- a) be available as documented information;
- b) be communicated within the organization;
- c) be available to interested parties, as appropriate.

5.3 Roles, responsibilities and authorities

Top management shall ensure that the responsibilities and authorities for relevant roles are assigned and communicated within the organization.

Top management shall assign the responsibility and authority for:

- a) ensuring that the BCMS conforms to the requirements of this document;
- b) reporting on the performance of the BCMS to top management.

6 Planning

6.1 Actions to address risks and opportunities

6.1.1 Determining risks and opportunities

When planning for the BCMS, the organization shall consider the issues referred to in [4.1](#) and the requirements referred to in [4.2](#) and determine the risks and opportunities that need to be addressed to:

- a) give assurance that the BCMS can achieve its intended outcome(s);
- b) prevent, or reduce, undesired effects;
- c) achieve continual improvement.

6.1.2 Addressing risks and opportunities

The organization shall plan:

- a) actions to address these risks and opportunities;
- b) how to:
 - 1) integrate and implement the actions into its BCMS processes (see [8.1](#));
 - 2) evaluate the effectiveness of these actions (see [9.1](#)).

NOTE Risks and opportunities relate to the effectiveness of the management system. Risks related to disruption of the business are addressed in [8.2](#).

6.2 Business continuity objectives and planning to achieve them

6.2.1 Establishing business continuity objectives

The organization shall establish business continuity objectives at relevant functions and levels.

The business continuity objectives shall:

- a) be consistent with the business continuity policy;
- b) be measurable (if practicable);
- c) take into account applicable requirements (see [4.1](#) and [4.2](#));
- d) be monitored;
- e) be communicated;
- f) be updated as appropriate.

The organization shall retain documented information on the business continuity objectives.

6.2.2 Determining business continuity objectives

When planning how to achieve its business continuity objectives, the organization shall determine:

- a) what will be done;
- b) what resources will be required;
- c) who will be responsible;
- d) when it will be completed;
- e) how the results will be evaluated.

6.3 Planning changes to the business continuity management system

When the organization determines the need for changes to the BCMS, including those identified in [Clause 10](#), the changes shall be carried out in a planned manner.

The organization shall consider:

- a) the purpose of the changes and their potential consequences;
- b) the integrity of the BCMS;
- c) the availability of resources;
- d) the allocation or reallocation of responsibilities and authorities.

7 Support

7.1 Resources

The organization shall determine and provide the resources needed for the establishment, implementation, maintenance and continual improvement of the BCMS.

7.2 Competence

The organization shall:

- a) determine the necessary competence of person(s) doing work under its control that affects its business continuity performance;
- b) ensure that these persons are competent on the basis of appropriate education, training, or experience;

- c) where applicable, take actions to acquire the necessary competence, and evaluate the effectiveness of the actions taken;
- d) retain appropriate documented information as evidence of competence.

NOTE Applicable actions can include, for example, the provision of training to, the mentoring of, or the re-assignment of currently employed persons; or the hiring or contracting of competent persons.

7.3 Awareness

Persons doing work under the organization's control shall be aware of:

- a) the business continuity policy;
- b) their contribution to the effectiveness of the BCMS, including the benefits of improved business continuity performance;
- c) the implications of not conforming with the BCMS requirements;
- d) their own role and responsibilities before, during and after disruptions.

7.4 Communication

The organization shall determine the internal and external communications relevant to the BCMS, including:

- a) on what it will communicate;
- b) when to communicate;
- c) with whom to communicate;
- d) how to communicate;
- e) who will communicate.

7.5 Documented information

7.5.1 General

The organization's BCMS shall include:

- a) documented information required by this document;
- b) documented information determined by the organization as being necessary for the effectiveness of the BCMS.

NOTE The extent of documented information for a BCMS can differ from one organization to another due to:

- the size of organization and its type of activities, processes, products and services, and resources;
- the complexity of processes and their interactions;
- the competence of persons.

7.5.2 Creating and updating

When creating and updating documented information the organization shall ensure appropriate:

- a) identification and description (e.g. a title, date, author, or reference number);
- b) format (e.g. language, software version, graphics) and media (e.g. paper, electronic);

- c) review and approval for suitability and adequacy.

7.5.3 Control of documented information

7.5.3.1 Documented information required by the BCMS and by this document shall be controlled to ensure:

- a) it is available and suitable for use, where and when it is needed;
- b) it is adequately protected (e.g. from loss of confidentiality, improper use, or loss of integrity).

7.5.3.2 For the control of documented information, the organization shall address the following activities, as applicable:

- a) distribution, access, retrieval and use;
- b) storage and preservation, including preservation of legibility;
- c) control of changes (e.g. version control);
- d) retention and disposition.

Documented information of external origin determined by the organization to be necessary for the planning and operation of the BCMS shall be identified, as appropriate, and controlled.

NOTE Access can imply a decision regarding the permission to view the documented information only, or the permission and authority to view and change the documented information.

8 Operation

8.1 Operational planning and control

The organization shall plan, implement and control the processes needed to meet requirements, and to implement the actions determined in [6.1](#), by:

- a) establishing criteria for the processes;
- b) implementing control of the processes in accordance with the criteria;
- c) keeping documented information to the extent necessary to have confidence that the processes have been carried out as planned.

The organization shall control planned changes and review the consequences of unintended changes, taking action to mitigate any adverse effects, as necessary.

The organization shall ensure that outsourced processes and the supply chain are controlled.

8.2 Business impact analysis and risk assessment

8.2.1 General

The organization shall:

- a) implement and maintain systematic processes for analysing the business impact and assessing the risks of disruption;
- b) review the business impact analysis and risk assessment at planned intervals and when there are significant changes within the organization or the context in which it operates.

NOTE The organization determines the order in which the business impact analysis and risk assessment are conducted.

8.2.2 Business impact analysis

The organization shall use the process for analysing business impacts to determine business continuity priorities and requirements. The process shall:

- a) define the impact types and criteria relevant to the organization's context;
- b) identify the activities that support the provision of products and services;
- c) use the impact types and criteria for assessing the impacts over time resulting from the disruption of these activities;
- d) identify the time frame within which the impacts of not resuming activities would become unacceptable to the organization;

NOTE 1 This time frame can be referred to as the "maximum tolerable period of disruption (MTPD)".

- e) set prioritized time frames within the time identified in d) for resuming disrupted activities at a specified minimum acceptable capacity;

NOTE 2 This time frame can be referred to as the "recovery time objective (RTO)".

- f) use this analysis to identify prioritized activities;
- g) determine which resources are needed to support prioritized activities;
- h) determine the dependencies, including partners and suppliers, and interdependencies of prioritized activities.

8.2.3 Risk assessment

The organization shall implement and maintain a risk assessment process.

NOTE The process for risk assessment is addressed in ISO 31000.

The organization shall:

- a) identify the risks of disruption to the organization's prioritized activities and to their required resources;
- b) analyse and evaluate the identified risks;
- c) determine which risks require treatment.

NOTE Risks in this subclause relate to the disruption of business activities. Risks and opportunities related to the effectiveness of the management system are addressed in [6.1](#).

8.3 Business continuity strategies and solutions

8.3.1 General

Based on the outputs from the business impact analysis and risk assessment, the organization shall identify and select business continuity strategies that consider options for before, during and after disruption. The business continuity strategies shall be comprised of one or more solutions.

8.3.2 Identification of strategies and solutions

Identification shall be based on the extent to which strategies and solutions:

- a) meet the requirements to continue and recover prioritized activities within the identified time frames and agreed capacity;

- b) protect the organization's prioritized activities;
- c) reduce the likelihood of disruption;
- d) shorten the period of disruption;
- e) limit the impact of disruption on the organization's products and services;
- f) provide for the availability of adequate resources.

8.3.3 Selection of strategies and solutions

Selection shall be based on the extent to which strategies and solutions:

- a) meet the requirements to continue and recover prioritized activities within the identified time frames and agreed capacity;
- b) consider the amount and type of risk the organization may or may not take;
- c) consider associated costs and benefits.

8.3.4 Resource requirements

The organization shall determine the resource requirements to implement the selected business continuity solutions. The types of resources considered shall include, but not be limited to:

- a) people;
- b) information and data;
- c) physical infrastructure such as buildings, workplaces or other facilities and associated utilities;
- d) equipment and consumables;
- e) information and communication technology (ICT) systems;
- f) transportation and logistics;
- g) finance;
- h) partners and suppliers.

8.3.5 Implementation of solutions

The organization shall implement and maintain selected business continuity solutions so they can be activated when needed.

8.4 Business continuity plans and procedures

8.4.1 General

The organization shall implement and maintain a response structure that will enable timely warning and communication to relevant interested parties. It shall provide plans and procedures to manage the organization during a disruption. The plans and procedures shall be used when required to activate business continuity solutions.

NOTE There are different types of procedures that comprise business continuity plans.

The organization shall identify and document business continuity plans and procedures based on the output of the selected strategies and solutions.

The procedures shall:

- a) be specific regarding the immediate steps that are to be taken during a disruption;
- b) be flexible to respond to the changing internal and external conditions of a disruption;
- c) focus on the impact of incidents that potentially lead to disruption;
- d) be effective in minimizing the impact through the implementation of appropriate solutions;
- e) assign roles and responsibilities for tasks within them.

8.4.2 Response structure

8.4.2.1 The organization shall implement and maintain a structure, identifying one or more teams responsible for responding to disruptions.

8.4.2.2 The roles and responsibilities of each team and the relationships between the teams shall be clearly stated.

8.4.2.3 Collectively, the teams shall be competent to:

- a) assess the nature and extent of a disruption and its potential impact;
- b) assess the impact against pre-defined thresholds that justify initiation of a formal response;
- c) activate an appropriate business continuity response;
- d) plan actions that need to be undertaken;
- e) establish priorities (using life safety as the first priority);
- f) monitor the effects of the disruption and the organization's response;
- g) activate the business continuity solutions;
- h) communicate with relevant interested parties, authorities and the media.

8.4.2.4 For each team there shall be:

- a) identified personnel and their alternates with the necessary responsibility, authority and competence to perform their designated role;
- b) documented procedures to guide their actions (see [8.4.4](#)), including those for the activation, operation, coordination and communication of the response.

8.4.3 Warning and communication

8.4.3.1 The organization shall document and maintain procedures for:

- a) communicating internally and externally to relevant interested parties, including what, when, with whom and how to communicate;

NOTE The organization can document and maintain procedures for how, and under what circumstances, the organization communicates with employees and their emergency contacts.

- b) receiving, documenting and responding to communications from interested parties, including any national or regional risk advisory system or equivalent;
- c) ensuring the availability of the means of communication during a disruption;

- d) facilitating structured communication with emergency responders;
- e) providing details of the organization's media response following an incident, including a communications strategy;
- f) recording the details of the disruption, the actions taken and the decisions made.

8.4.3.2 Where applicable, the following shall also be considered and implemented:

- a) alerting interested parties potentially impacted by an actual or impending disruption;
- b) ensuring appropriate coordination and communication between multiple responding organizations.

The warning and communication procedures shall be exercised as part of the organization's exercise programme described in [8.5](#).

8.4.4 Business continuity plans

8.4.4.1 The organization shall document and maintain business continuity plans and procedures. The business continuity plans shall provide guidance and information to assist teams to respond to a disruption and to assist the organization with response and recovery.

8.4.4.2 Collectively, the business continuity plans shall contain:

- a) details of the actions that the teams will take in order to:
 - 1) continue or recover prioritized activities within predetermined time frames;
 - 2) monitor the impact of the disruption and the organization's response to it;
- b) reference to the pre-defined threshold(s) and process for activating the response;
- c) procedures to enable the delivery of products and services at agreed capacity;
- d) details to manage the immediate consequences of a disruption giving due regard to:
 - 1) the welfare of individuals;
 - 2) the prevention of further loss or unavailability of prioritized activities;
 - 3) the impact on the environment.

8.4.4.3 Each plan shall include:

- a) the purpose, scope and objectives;
- b) the roles and responsibilities of the team that will implement the plan;
- c) actions to implement the solutions;
- d) supporting information needed to activate (including activation criteria), operate, coordinate and communicate the team's actions;
- e) internal and external interdependencies;
- f) the resource requirements;
- g) the reporting requirements;
- h) a process for standing down.

Each plan shall be usable and available at the time and place at which it is required.

8.4.5 Recovery

The organization shall have documented processes to restore and return business activities from the temporary measures adopted during and after a disruption.

8.5 Exercise programme

The organization shall implement and maintain a programme of exercising and testing to validate over time the effectiveness of its business continuity strategies and solutions.

The organization shall conduct exercises and tests that:

- a) are consistent with its business continuity objectives;
- b) are based on appropriate scenarios that are well planned with clearly defined aims and objectives;
- c) develop teamwork, competence, confidence and knowledge for those who have roles to perform in relation to disruptions;
- d) taken together over time, validate its business continuity strategies and solutions;
- e) produce formalized post-exercise reports that contain outcomes, recommendations and actions to implement improvements;
- f) are reviewed within the context of promoting continual improvement;
- g) are performed at planned intervals and when there are significant changes within the organization or the context in which it operates.

The organization shall act on the results of its exercising and testing to implement changes and improvements.

8.6 Evaluation of business continuity documentation and capabilities

The organization shall:

- a) evaluate the suitability, adequacy and effectiveness of its business impact analysis, risk assessment, strategies, solutions, plans and procedures;
- b) undertake evaluations through reviews, analysis, exercises, tests, post-incident reports and performance evaluations;
- c) conduct evaluations of the business continuity capabilities of relevant partners and suppliers;
- d) evaluate compliance with applicable legal and regulatory requirements, industry best practices, and conformity with its own business continuity policy and objectives;
- e) update documentation and procedures in a timely manner.

These evaluations shall be conducted at planned intervals, after an incident or activation, and when significant changes occur.

9 Performance evaluation

9.1 Monitoring, measurement, analysis and evaluation

The organization shall determine:

- a) what needs to be monitored and measured;

- b) the methods for monitoring, measurement, analysis and evaluation, as applicable, to ensure valid results;
- c) when and by whom the monitoring and measuring shall be performed;
- d) when and by whom the results from monitoring and measurement shall be analysed and evaluated.

The organization shall retain appropriate documented information as evidence of the results.

The organization shall evaluate the BCMS performance and the effectiveness of the BCMS.

9.2 Internal audit

9.2.1 General

The organization shall conduct internal audits at planned intervals to provide information on whether the BCMS:

- a) conforms to:
 - 1) the organization's own requirements for its BCMS;
 - 2) the requirements of this document;
- b) is effectively implemented and maintained.

9.2.2 Audit programme(s)

The organization shall:

- a) plan, establish, implement and maintain an audit programme(s) including the frequency, methods, responsibilities, planning requirements and reporting, which shall take into consideration the importance of the processes concerned and the results of previous audits;
- b) define the audit criteria and scope for each audit;
- c) select auditors and conduct audits to ensure objectivity and the impartiality of the audit process;
- d) ensure that the results of the audits are reported to relevant managers;
- e) retain documented information as evidence of the implementation of the audit programme(s) and the audit results;
- f) ensure that any necessary corrective actions are taken without undue delay to eliminate detected nonconformities and their causes;
- g) ensure that follow-up audit actions include the verification of the actions taken and the reporting of verification results.

9.3 Management review

9.3.1 General

Top management shall review the organization's BCMS, at planned intervals, to ensure its continuing suitability, adequacy and effectiveness.

9.3.2 Management review input

The management review shall include consideration of:

- a) the status of actions from previous management reviews;

- b) changes in external and internal issues that are relevant to the BCMS;
- c) information on the BCMS performance, including trends in:
 - 1) nonconformities and corrective actions;
 - 2) monitoring and measurement evaluation results;
 - 3) audit results;
- d) feedback from interested parties;
- e) the need for changes to the BCMS, including the policy and objectives;
- f) procedures and resources that could be used in the organization to improve the BCMS' performance and effectiveness;
- g) information from the business impact analysis and risk assessment;
- h) output from the evaluation of business continuity documentation and capabilities (see [8.6](#));
- i) risks or issues not adequately addressed in any previous risk assessment;
- j) lessons learned and actions arising from near-misses and disruptions;
- k) opportunities for continual improvement.

9.3.3 Management review outputs

9.3.3.1 The outputs of the management review shall include decisions related to continual improvement opportunities and any need for changes to the BCMS to improve its efficiency and effectiveness, including the following:

- a) variations to the scope of the BCMS;
- b) update of the business impact analysis, risk assessment, business continuity strategies and solutions, and business continuity plans;
- c) modification of procedures and controls to respond to internal or external issues that may impact the BCMS;
- d) how the effectiveness of controls will be measured.

9.3.3.2 The organization shall retain documented information as evidence of the results of management reviews. It shall:

- a) communicate the results of the management review to relevant interested parties;
- b) take appropriate action relating to those results.

10 Improvement

10.1 Nonconformity and corrective action

10.1.1 The organization shall determine opportunities for improvement and implement necessary actions to achieve the intended outcomes of its BCMS.

10.1.2 When a nonconformity occurs, the organization shall:

- a) react to the nonconformity, and, as applicable:
 - 1) take action to control and correct it;
 - 2) deal with the consequences;
- b) evaluate the need for action to eliminate the cause(s) of the nonconformity, in order that it does not recur or occur elsewhere, by:
 - 1) reviewing the nonconformity;
 - 2) determining the causes of the nonconformity;
 - 3) determining if similar nonconformities exist, or can potentially occur;
- c) implement any action needed;
- d) review the effectiveness of any corrective action taken;
- e) make changes to the BCMS, if necessary.

Corrective actions shall be appropriate to the effects of the nonconformities encountered.

10.1.3 The organization shall retain documented information as evidence of:

- a) the nature of the nonconformities and any subsequent actions taken;
- b) the results of any corrective action.

10.2 Continual improvement

The organization shall continually improve the suitability, adequacy and effectiveness of the BCMS, based on qualitative and quantitative measures.

The organization shall consider the results of analysis and evaluation, and the outputs from management review, to determine if there are needs or opportunities, relating to the business, or to the BCMS, that shall be addressed as part of continual improvement.

NOTE The organization can use the processes of the BCMS, such as leadership, planning and performance evaluation, to achieve improvement.



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- [7] ISO 22316, *Security and resilience — Organizational resilience — Principles and attributes*
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- [15] ISO 31000, *Risk management — Guidelines*
- [16] IEC 31010, *Risk management — Risk assessment techniques*
- [17] ISO Guide 73, *Risk management — Vocabulary*

**Water quality — Evaluation of
genotoxicity by measurement of the
induction of micronuclei —**

**Part 2:
Mixed population method using the cell
line V79**

*Qualité de l'eau — Évaluation de la génotoxicité par le mesurage de
l'induction de micronoyaux —*

*Partie 2: Méthode de la population mélangée à l'aide de la lignée de
cellules V79*



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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 21427-2 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

ISO 21427 consists of the following parts, under the general title *Water quality — Evaluation of genotoxicity by measurement of the induction of micronuclei*:

- *Part 1: Evaluation of genotoxicity using amphibian larvae*
- *Part 2: Mixed population method using the cell line V79*

Part 2: Mixed population method using the cell line V79

IMPORTANT — It is absolutely essential that tests conducted according to this part of ISO 21427 be carried out by suitably trained staff.

The assay is based on the increase in the frequency of micronucleated cells after incubation with and without metabolic activation.

ISO 5667-16, Water quality — Sampling — Part 16: Guidance on biotesting of samples

aqueous solution of chemicals (e.g. NADP, Glucose-6-phosphate and inorganic salts) needed for the activity of the enzymes in the S9 fraction

3.3

dilution level D

denominator of the dilution coefficient (using the numerator 1) of a mixture of water or waste water with dilution water as integral number

NOTE For undiluted water or waste water, this coefficient is per definition 1:1. The corresponding smallest possible D value is 1.

3.4

D value

smallest value of D at which, under the conditions of this part of ISO 21427, no increase in the number of micronuclei per culture is detected

NOTE In the case of more than one D value (at maximum two are possible, see 9.2), the highest D value is decisive.

3.5

karyotype

characteristic of the nucleus of a cell, including its size, form and chromosome number

3.6

micronuclei

small particles consisting of acentric fragments of chromosomes and/or entire chromosomes which lag behind at anaphase stage of cell division and form, after telophase, single or multiple micronuclei in the cytoplasm

3.7

mitotic index

percentage of cells of a cell population under division at a particular time of observation

3.8

plating efficiency

measure of the number of colonies originated from single cells

3.9

proliferation index

rate at which cells are dividing within the culture

3.10

proliferation rate

rate with which cells replicate, calculated by a formula which takes into account 1, 2, 4 and 8 cell stages of clones

3.11

S9 fraction

9 000 g supernatant of a tissue homogenate in 0,15 mol/l KCl, obtained from livers of male rats (200 g to 300 g) pretreated with an appropriate substance or substance combination for enzyme induction

3.12

S9 mix

mixture of the S9 fraction and the cofactor solution

3.13

stock culture

frozen culture for the preservation of the characteristics of V79 cells

3.14

survival index

percentage of surviving cells compared to all cells, used as index of toxicity

3.15

test culture

culture of cells used for the study

4 Principle

The possible clastogenic and/or aneugenic activity of the test sample is detected by comparing, for the respective activation condition, the number of micronucleated cells in cultures treated with the negative control and the number in cultures treated with undiluted and diluted test samples, respectively.

During cell division, chromatid fragments without centromeres will not move to the nuclei of the daughter cells and will stay within the cytoplasm. Part of the chromosome aberrations induced by the test item will be chromatid fragments without centromeres and will, therefore, not be incorporated in the nuclei of the daughter cells. In addition, spindle disorders may lead to chromosomes which are not incorporated into the nucleus. These particles will form micronuclei in the plasma.

V79 cells are exposed for 24 h (4 h with the S9 mix) to a range of concentrations of a test sample. Thereafter, slides are prepared, and cells are stained and evaluated for the presence of micronucleated cells. An increased incidence of these micronucleated cells in comparison to the negative control indicates that the test item may cause chromosome breaks or spindle disorders in V79 cells *in vitro*.

5 Interferences

Biologically relevant alterations of the culture conditions may induce chromosome aberration due to secondary mechanisms resulting in artificial positive and, therefore, irrelevant results^[16]. Those factors are, e.g. stronger changes in osmolality or pH, precipitation of test sample and phagocytosis thereof, and strong cytotoxic effects of the test sample. Therefore, test samples should be monitored at least for changes in pH or osmolality of the cultures using the same proportion of test item per culture as will be used later under test conditions. If there is a shift in pH in the culture, the test item should be adjusted to $\text{pH } 7,0 \pm 0,2$. If there is a change in osmolality, the highest concentration used in the test has to be reduced so that no relevant alteration of osmolality occurs in the cultures. To avoid artifacts based on phagocytosis or severe cytotoxicity, limitations are given for the highest concentration, which should be used for testing (see 9.1 and 9.2).

6 Reagents and media

As far as possible use "reagent grade" chemicals.

If chemicals with different amounts of water of crystallization are used, calculate the needed amounts accordingly.

Always perform autoclaving for 20 min at $121\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$. Cover vessels loosely (e.g. with aluminium foil). Never seal air-tight.

6.1 Water.

Prepare all aqueous solutions with water of a conductivity of $\leq 5\text{ }\mu\text{S/cm}$.

6.2 Reagents.

6.2.1 Glucose-6-phosphate dihydrate, $\text{C}_6\text{H}_{11}\text{O}_9\text{PNa}_2 \cdot 2\text{H}_2\text{O}$.

6.2.2 Nicotinamide adenine dinucleotide phosphate disodium salt, NADP, $\text{C}_{21}\text{H}_{26}\text{N}_7\text{Na}_2\text{O}_{17}\text{P}_3$.

6.2.3 Magnesium chloride hexahydrate, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$.

6.2.4 Potassium dihydrogenphosphate, KH_2PO_4 .

6.2.5 di-Sodium hydrogenphosphate dihydrate, $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$.

6.2.6 Ethanol (absolute), $\text{C}_2\text{H}_5\text{OH}$.

6.2.7 Glacial acetic acid, CH_3COOH .

6.2.8 Formaldehyde, HCHO , 37 % volume fraction.

6.2.9 tri-Sodium citrate dihydrate, $\text{HOC}(\text{COONa})(\text{CH}_2\text{COONa})_2 \cdot 2 \text{H}_2\text{O}$.

6.2.10 di-Sodium hydrogenphosphate, Na_2HPO_4 .

6.2.11 Sodium dihydrogenphosphate, NaH_2PO_4 .

6.2.12 May-Grünwald-solution, modified ¹⁾.

6.2.13 Hydrochloric acid, $c(\text{HCl}) = 1 \text{ mol/l}$.

6.2.14 Sodium hydroxide solution, $c(\text{NaOH}) = 1 \text{ mol/l}$.

6.2.15 Dimethyl sulfoxide (DMSO), $\text{C}_2\text{H}_6\text{SO}_4$.

6.2.16 Positive controls.

6.2.16.1 Cyclophosphamide, monohydrate, $\text{C}_7\text{H}_{15}\text{Cl}_2\text{N}_2\text{O}_2\text{P} \cdot \text{H}_2\text{O}$.

CAS Registration No: 6055-19-2.

6.2.16.2 Ethyl-methane sulfonate (EMS), $\text{CH}_3\text{SO}_3\text{CH}_2\text{CH}_3$.

CAS Registration No: 62-50-0.

6.2.17 Sodium citrate solution for hypotonic treatment.

Prepare a 1,5 % solution of tri-sodium citrate in water.

6.2.18 Fixation solution.

Mix 50 ml of glacial acetic acid with 150 ml of ethanol, add 2,5 ml of a 37 % formaldehyde solution.

6.2.19 Buffer solution according to WEISE (pH 7,2) ¹⁾.

This solution is commercially available in ampoules. Dilute the contents of one ampoule in water and, using a 1 000 ml measuring flask, bring to volume with water.

6.2.20 Giemsa solution ¹⁾.

Prepare a 2,6 % Giemsa solution in buffer according to WEISE (pH 7,2) (6.2.19). Filter prior to use.

6.2.21 Phosphate buffer.

Dissolve 2,13 g of Na_2HPO_4 in 1 l water. Dissolve 1,8 g of NaH_2PO_4 in 1 l water. Mix both solutions at the ratio of 4:1 and adjust to a final pH of 7,4.

6.2.22 MEM-medium (= Minimal Essential Medium) with stabilized glutamine ¹⁾.

6.2.23 Fetal bovine serum (= FCS) ¹⁾.

¹⁾ This reagent is commercially available. This information is given for the convenience of users of this part of ISO 21427 and does not constitute an endorsement by ISO of these products.

6.2.24 Penicillin/Streptomycin solution, 10 000 E/10 000 µg/ml ¹⁾.

6.2.25 Amphotericin-B solution, 250 µg/ml ¹⁾.

6.2.26 Trypsin/EDTA solution, 0,25 % ¹⁾.

6.2.27 Hanks' Balanced Salt Solution (= HBSS) ¹⁾.

6.2.28 Hanks' Balanced Salt Solution (= HBSS) without Ca²⁺ and Mg²⁺ ¹⁾.

6.2.29 Potassium chloride solution.

Dissolve 4 g of potassium chloride, in 1 l water.

6.3 Preparation of culture media

6.3.1 Culture medium with FCS.

This medium is used as general culture medium and for treatment of cells without the S9 mix.

Mix 500 ml of MEM-medium, 50 ml of FCS, 5 ml of Penicillin/Streptomycin solution and 5 ml of Amphotericin-B solution.

The medium is stable for up to 4 weeks if stored in a refrigerator at 4 °C ± 2 °C.

6.3.2 Culture medium without FCS.

This medium is used only for the treatment period of cells under activation condition (S9 mix).

Mix 500 ml of MEM-medium, 5 ml of Penicillin/Streptomycin solution and 5 ml of Amphotericin-B solution.

The medium is stable for up to 4 weeks if stored in a refrigerator at 4 °C ± 2 °C.

6.4 Cell system.

6.4.1 Cell line, storage

The V79 cell line is a permanent cell line of Chinese hamster lung cells with

- a high proliferation rate (cell cycle length about 12 h to 16 h);
- a high plating efficiency (≥ 90 %);
- a stable karyotype (modal number of chromosomes = 22).

Store permanent cultures (1 ml samples including 7 % DMSO) in liquid nitrogen at about –196 °C. Prior to freezing, check each batch for mycoplasma contamination. Karyotype and plating efficiency (colony-forming ability) should be determined at least prior to the first use of a thawed culture.

6.4.2 Cultivation

To start a culture, thaw a permanent culture in a water bath at 37 °C and add 0,5 ml of this sample to a 25 cm² culture flask filled already with approximately 5 ml of MEM (minimal essential medium; composed of medium, glutamine and antibiotics) including 10 % FCS (fetal calf serum). Cultivate the cells at 37 °C, using 5 % carbon dioxide and a humidity of at least 90 %. Subcultivate the cells twice a week.

Withdraw the flasks (25 cm²) from the incubator and place them on a clean bench. Open the flasks singly and remove the medium by suction. Wash the cells once with 5 ml Hanks Balanced Salt Solution (HBBS, without Ca²⁺ and Mg²⁺) for about 5 min. Thereafter, remove the medium again.

Trypsinize the cells for about 5 min using about 1,0 ml of trypsin (0,25 %) and approximately 1,0 ml HBBS (without Ca²⁺ and Mg²⁺) to separate the cells from the bottom of the culture flask.

Stop this reaction by adding approximately 3 ml of MEM including 10 % FCS.

Pipette this mixture several times to separate the cells from the flask and to obtain homogenous single cell suspensions.

Count the number of cells in a 10 µl sample in a hemocytometer ²⁾.

Dilute the suspension to the required cell density (30 000 to 80 000 per culture) using MEM including 10 % of FCS.

6.4.3 Duration of cell cycle

The cell cycle length of the V79 cells is normally about 12 h to 16 h. Determine its laboratory specific length using the BrdU³⁾ method (see Annex A).

6.5 Metabolic activation

6.5.1 S9 fraction

For the treatment of enzyme induction and preparation of the S9 fraction, see Annex C. If the S9 fraction is commercially purchased, it shall have been prepared (including enzyme induction) according to Annex C.

6.5.2 S9 mix

Prepare the needed amount of the S9 fraction freshly on the day of test or, if stored frozen, thaw at room temperature. Immediately thereafter, prepare the S9 mix by mixing the following compounds under sterile conditions:

- a) 1 aliquot of S9 fraction;
- b) 9 aliquots of S9 supplement (cofactor solution).

Keep the S9 mix permanently on ice (e.g. in a double-walled separator funnel containing iced water in between these walls) and use it only on the same day. At the end of this day, discard the remaining S9 mix. The concentrations of cofactors in the S9 mix are as follows:

MgCl ₂	8 mmol/l
KCl	33 mmol/l
Glucose-6-phosphate	5 mmol/l
NADP	4 mmol/l
Phosphate buffer (pH 7,4)	15 mmol/l

2) Alternatively, an automatic cell or particle counter device may be used.

3) BrdU stands for bromodeoxyuridine.

7 Apparatus

- 7.1 Cryo-vials, 1 ml, 2 ml, 5 ml.
- 7.2 Cell culture flasks, 25 cm², 75 cm².
- 7.3 Culture chambers, appropriate for 4 microscope slides, approx. 9 cm × 13 cm.
- 7.4 Microscope slides with frosted part.
- 7.5 CO₂ incubator.
- 7.6 Laminar-airflow work bench.
- 7.7 Water bath.
- 7.8 Vacuum pump.
- 7.9 Inverse microscope.
- 7.10 Light microscope.
- 7.11 Bunsen burner.
- 7.12 Centrifuge.
- 7.13 Freezer (−80 °C).
- 7.14 Analytical balance.
- 7.15 Glass pipettes, 1 ml, 5 ml, 10 ml.
- 7.16 Adjustable volume pipettes.
- 7.17 Neubauer counting chamber.
- 7.18 Photographic clips.
- 7.19 Dewar tank for storage of the cells in liquid nitrogen.
- 7.20 Tweezers.
- 7.21 Cuvettes including holders for staining.
- 7.22 Sterile filters, 0,22 µm.

8 Test facility criteria

The test facility is qualified for the execution of this part of ISO 21427 if the *in vitro* micronucleus test is established in this facility according to the following criteria:

- a couple of independent experiments shall already have been performed;
- a couple of known mutagenic and non-mutagenic chemicals shall already have been tested.

It should be decided case by case whether and to what extent additional instructions may be necessary for the application of this part of ISO 21427.

9 Procedure

9.1 Sampling and samples

Test samples as soon as possible after sampling, i.e. on the day of collection. Divide large samples into appropriate portions in advance, since thawed samples may only be used on the same day as they were thawed.

High or low pH-values of the test sample may trigger cell toxic effects lowering the possible maximum testable concentration. Therefore, pH of the test sample should be monitored and adjusted, if necessary, to $\text{pH } 7,0 \pm 0,2$ using either HCl or NaOH solution (6.2.13 and 6.2.14). Select the concentrations of acid or alkali such that the added volumes are as small as possible. Avoid over-titration. The change of the sample pH and the resulting effects shall be taken into consideration (see ISO 5667-16).

Shake test samples thoroughly before use.

Centrifuge the samples containing solids and use only the liquid supernatant for further processing.

Sterilize all the samples by filtration through sterile filters (7.22). Do not extract or concentrate the samples.

NOTE 1 If necessary, keep the samples at 0 °C to 5 °C for up to 48 h and below –18 °C for up to two months, respectively.

NOTE 2 See ISO 5667-16.

If dilutions are necessary, perform these with sterilized water (6.1).

9.2 Experimental size

Use two cultures per experimental group. Evaluate 1 000 cells per culture for micronuclei. See Table 1.

Table 1 — Experimental size

Treatment group	S9 mix	Treatment time h	Time of harvest h
negative control dilution water	-	24	24
undiluted test item ^a	-	24	24
1:2	-	24	24
1:4	-	24	24
1:8	-	24	24
1:16	-	24	24
1:32	-	24	24
positive control EMS (6.2.16.2) 350 µg/ml	-	24	24
negative control dilution water	+	4	24
undiluted test item ^a	+	4	24
1:2	+	4	24
1:4	+	4	24
1:8	+	4	24
1:16	+	4	24
1:32	+	4	24
positive control cyclophosphamide (6.2.16.1) 2,5 µg/ml	+	4	24
^a If osmolality is relevantly altered, the highest dilution which does not alter osmolality of the culture.			

9.3 Negative controls

Treat the cultures of the negative controls with the dilution water in the same volume as other cultures are treated with test item.

9.4 Positive controls

Dissolve the positive controls in MEM to result in a concentration of EMS of 1,75 mg/ml and in a concentration of cyclophosphamide of 12,5 µg/ml. Apply a volume of 1 ml per culture.

Store the stock solutions of the positive controls at –80 °C. In this case, they should be thawed shortly prior to treatment.

9.5 Time schedule

– 6 h	Seeding of cells
0 h	Treatment
+ 4 h	Washing of cultures (only those with S9 mix)
+ 24 h	Harvest and slide preparation

9.6 Preparatory steps

Thaw permanent cultures in a water bath at 37 °C. Add 0,5 ml of a thawed culture to a 25 cm² flask which already contains 5 ml of MEM (including 10 % FCS). Incubate cultures at 37 °C, using an atmosphere containing a volume fraction of 5 % CO₂ and at least 90 % humidity, to reach a confluency (contact inhibition) of 50 % to maximally 100 % (about 2 d to 4 d). To remove dead cells, replace the medium about 30 h after culture initiation. Use the remaining cells for experiments. (They may also be used for further subculturing.) Discard cells which have undergone more than 15 passages because they cannot be used for further experiments.

9.7 Preparation of test cultures

9.7.1 General

Wash the cells once with 5 ml HBBS (without Ca²⁺ and Mg²⁺) for about 5 min and then remove the medium by suction.

Trypsinize the cells for about 5 min using about 1,0 ml of trypsin (0,25 %) and approximately 1,0 ml of HBBS (without Ca²⁺ and Mg²⁺) to separate the cells from the bottom of the culture flask.

Stop this reaction by adding approximately 3 ml of MEM including 10 % FCS.

Pipette this mixture several times to separate cells from the flask and to obtain homogenous, single cell suspensions.

Count the number of cells in a 10 µl sample in a hemocytometer ⁴⁾.

Dilute the suspension to the required cell density (30 000 to 80 000 per 5 ml culture) using MEM including 10 % FCS.

9.7.2 Seeding

Add 5 ml of cell suspension to each chamber of a culture dish (7.3), of which each chamber contains a slide. Each chamber represents one culture.

Incubate the dishes for 6 h at 37 °C, in an atmosphere containing a volume fraction of 5 % CO₂ and at least 90 % humidity.

Prior to treatment, check the dishes visually for cell attachment. (See Annex B.)

9.7.3 Preparation of S9 mix

Prepare the S9 mix according to 6.5.2.

9.8 Treatment of test cultures

9.8.1 Treatment without S9 mix

Remove the medium from the culture chambers approximately 6 h after seeding. Add 4 ml of fresh medium (with FCS). Thereafter, add the test sample, then either the positive control or the dilution water for the negative control in a volume of 1 ml (final volume of 5 ml). Incubate the cultures for 24 h at 37 °C, in an atmosphere containing a volume fraction of 5 % CO₂ volume fraction and at least 90 % humidity. Higher concentrations of test sample can be applied as long as the validity and cytotoxicity criteria (3.14, 9.8.5, 10.2) are met. If higher amounts of sample are used, the concentration of the medium has to be adjusted.

4) Alternatively, an automatic cell or particle counter device may be used.

9.8.2 Treatment with S9 mix

Remove the medium from the chambers approximately 6 h after seeding. Add to each chamber 1 ml of the S9 mix and 3 ml of fresh medium (without FCS). Thereafter, add the test sample, then either the positive control or the dilution water for the negative control in a volume of 1 ml per culture, resulting in a final volume of 5 ml. Incubate cultures for 4 h at 37 °C, in an atmosphere containing a volume fraction of 5 % CO₂ and at least 90 % humidity. Higher concentrations of test sample can be applied as long as the validity and cytotoxicity criteria (3.14, 9.8.5, 10.2) are met. If higher amounts of sample are used, the concentration of the medium has to be adjusted.

After 4 h, remove the medium and wash the cultures twice with 5 ml HBSS. Add 5 ml of MEM (incl. 10 % FCS) and incubate for further 20 h at 37 °C, in an atmosphere containing a volume fraction of 5 % CO₂ and at least 90 % humidity.

9.8.3 Processing of cell cultures

Prepare cells as follows ^[13]:

- remove the culture medium completely;
- treat each culture for 3 min with 5 ml of sodium citrate-solution (6.2.17) at a temperature of 37 °C (hypotonic treatment);
- remove the sodium citrate-solution and replace it by approximately 5 ml of fixation solution (6.2.18) at a temperature of about 4 °C for about 5 min; remove and replace the fixation solution by the same amount of fresh fixation solution (4 °C) for about 5 min;
- remove the slides from the culture chambers. Allow the fixation solution to drip off and dry the slides at ambient temperature.

9.8.4 Staining

- After the slides have dried, stain for 3 min in May-Grünwald-solution, modified (6.2.12);
- rinse the slides with WEISE-buffer (6.2.19) and place the slides for approximately 20 min in a Giemsa-solution (6.2.20);
- rinse twice with WEISE-buffer and remove surplus of staining material in xylene. Thereafter, cover the slides with coverslips and examine the slides as in 10.3.

9.8.5 Treatment for determination of the survival index

Seed approximately 250 000 cells into 25 cm² flasks already containing 5 ml of MEM (including 10 % FCS) per negative control or test sample concentration. Cultivate the cells for 30 h at 37 °C in an atmosphere containing a volume fraction of 5 % CO₂ and at least 90 % humidity (9.6). Thereafter, treat the cells with dilution water or test sample concentrations (9.8).

At the end of the respective treatment period, harvest the cells of all cultures as follows:

- remove the medium and wash once with 5 ml of HBBS (without Ca²⁺ and Mg²⁺);
- trypsinize with about 1,0 ml of trypsin (0,25 %) and about 1,0 ml of HBBS (without Ca²⁺ and Mg²⁺) to remove cells from bottom of flasks;
- stop the process by adding 3 ml of MEM (including 10 % FCS);
- pipette this mixture several times to separate cells from the flask and to obtain homogeneous single cell suspensions;

- dilute the cells to an appropriate amount in negative controls. Dilute all other cultures as cell suspensions of the negative controls were diluted. The same amount is taken from each culture and counted using a haemocytometer⁵⁾ to determine cell survival.

10 Evaluation and assessment

10.1 Cell morphology

At the end of the treatment, check all the cultures by an inverse microscope for changes in cell morphology and attachment of cells at a magnification of about 200-fold with the exception of positive controls. (See Annex B.)

10.2 Selection of concentrations to be analysed

For non-toxic samples, read only the highest tested concentration. For toxic samples, the lowest concentration which induces more than 50 % cytotoxicity in at least one of the indices (10.4, 10.5, 10.6) shall be chosen for reading of slides. Independently, no relevant changes should be induced in cell morphology (10.1) or attachment of cells. If those effects are observed for this concentration, the concentration for reading shall be reduced to the highest concentration which does not induce changes in cell morphology or cell attachment. For toxic samples, the next two lower dilutions of the test sample shall also be read. If a relevant increase in micronucleated cells is observed, lower concentrations are read until the first concentration without clastogenic effect is reached.

Prior to reading slides, check them for their quality. Use only slides of good quality for reading to avoid interference of slide quality with the assessment.

10.3 Reading of slides

Read 1 000 cells per culture and note the number of micronucleated cells amongst them.

Take into account the following criteria:

- the maximum size of a micronucleus is about 30 % of the size of a normal nucleus;
- concerning their staining micronucleus and nucleus shall have the same appearance;
- micronuclei shall be clearly separated from the nucleus;
- only cells with good cytoplasmic outlines are used for reading.

In addition to micronucleated cells, record fragmentations (cells with fragmented nuclei, cells with several nuclei of the same size, and cells with more than six micronuclei) amongst the evaluated 1 000 cells.

10.4 Mitotic index

Determine the mitotic index (3.7) for all cultures microscopically on the slides. The number of mitotic cells among a total of 1 000 cells per culture is determined. All cells which were not in interphase are defined as mitotic.

5) Alternatively, an automatic cell or particle counter device may be used.

10.5 Survival index

Determine the survival index (3.14 and 9.8.5) for all cultures except for positive controls. The number of cells observed for negative controls is set to 100 %. On this basis, the number of living cells in the test sample-treated cultures is converted to a percentage.

10.6 Proliferation index

Determine the proliferation index (3.9) for all cultures except for positive controls.

Evaluate 1 000 cells (counting all cells of each clone) per culture, separated into different clone sizes (1, 2, 4 or 8 cells). Calculate the proliferation index following Equation (1):

$$I_P = \frac{(n_{CL1} \times 1) + (n_{CL2} \times 2) + (n_{CL4} \times 3) + (n_{CL8} \times 4)}{n_{CL}} \quad (1)$$

where

- I_P is the proliferation index;
- n_{CL} is the number of clones counted;
- n_{CL1} is the number of clones with 1 cell;
- n_{CL2} is the number of clones with 2 cells;
- n_{CL4} is the number of clones with 3 or 4 cells;
- n_{CL8} is the number of clones with 5, 6, 7 or 8 cells.

Due to clone disintegration in experiments with the S9 mix, this index is most frequently smaller if compared to experiments without the S9 mix.

10.7 Validity criteria

The mean of the ratio of micronucleated cells in negative control cultures shall not exceed 3 %.

Positive controls shall have induced statistically significant increases in the ratio of micronucleated cells.

10.8 Assessment criteria

The following criteria shall be met for a positive result:

- a significant increase according to an appropriate statistical test (chi-squared test corrected for continuity according to Yates) [15] in micronucleated cells in treated cultures as compared to the respective negative controls;
- the number of micronucleated cells exceed the range of the historical negative control data.

If neither criteria is met, the test sample shall be evaluated as non-genotoxic according to this part of ISO 21427. If only one criterion is met, the test shall be repeated as the result is ambiguous.

10.9 Determination of the decisive D value

The “decisive D value” means the lowest D value (3.4) at which no detectable genotoxic effects are found for cultures treated with the test sample or dilutions thereof according to the criteria given in 10.8.

11 Precision

Results of an interlaboratory trial are given in Annex D.

12 Test report

This clause specifies which information is to be included in the test report. The clause shall require information to be given on at least the following aspects of the test:

- a reference to this part of ISO 21427 (ISO 21427-2);
- identity of the test sample (origin and date of sampling, pH value);
- result, according to Clause 10;
- if appropriate, any deviation from this procedure or circumstances that may have affected the result.

The documentation will include the following information:

- a) positive controls (chemical name, source, batch number or comparable data (if available));
- b) storage of sample and preparation of test sample (storage conditions (if not tested directly), adjustment of pH value, centrifugation (including *g* and time), filtration (including filter material and diameter of pores) and other manipulations);
- c) cell line (cell line, source, date of arrival, storage conditions, check for karyotype stability, plating efficiency and mycoplasma contamination);
- d) metabolizing system (preparation and origin of the S9 fraction, protein content, date of preparation, storage conditions);
- e) test environment (address of performing laboratory, date of test);
- f) results:
 - 1) survival index, mitotic index, proliferation index, number of cells analysed, micronuclei per culture including means, criteria for considering results positive, negative or equivocal, signs of toxicity, dose-response relationship, and
 - 2) where possible, statistical analysis and method applied, concurrent and historical negative-control data, concurrent and historical positive-control data, conclusions, D values, other observations (e.g. precipitation).

Annex A (informative)

Bromodeoxyuridine (BrdU) method

A.1 General

Incubate cells in a surplus of bromodeoxyuridine (BrdU) for about 14 h. Bromodeoxyuridine (BrdU) is incorporated into DNA as replacement for thymidine and results in an altered chromosome staining of affected chromatid parts. Using a special staining procedure, it is possible to differentiate between metaphases, which have undergone 1st, 2nd or 3rd mitosis since co-culturing with BrdU.

A.2 BrdU treatment

Perform subculturing in culture dishes by seeding approximately 80 000 cells of a single cell suspension per culture. Use four harvest times and four cultures per harvest time. Dissolve the BrdU freshly prior to use under light protection at a concentration of 5 µg of BrdU per millilitre in culture medium (including 10 % FCS) and store it under light protection at 4 °C until use.

24 h after passaging, remove the medium from the cultures and replace it under light protection by the “BrdU-medium”. Incubate the cultures for 14 h at 37 °C, in an atmosphere containing a volume fraction of 5 % CO₂ and at least 90 % humidity.

Thereafter, remove the medium under light protection and rinse twice with 5 ml HBSS. Add 5 ml of MEM (including 10 % FCS) per culture and incubate at 37 °C, in an atmosphere containing a volume fraction of 5 % CO₂ and a humidity of at least 90 % until harvest. Harvest the cultures 16 h, 18 h, 20 h and 22 h after the addition of BrdU.

A.3 Preparation of slides

Remove the culture medium completely.

Treat each culture for approximately 20 min with approximately 5 ml of a potassium chloride solution (6.2.29) at a temperature of approximately 37 °C (hypotonic treatment).

Add approximately 5 ml fixation solution (4 °C) (6.2.18) for about 15 min. Remove and replace the fixation solution by the same amount of fresh fixation solution (4 °C) for about 10 min. Remove and replace the fixation solution by the same amount of fresh fixation solution (4 °C) for about 5 min.

Remove the slides from the culture chambers. Allow the fixation solution to drip off and move the slide for a short period through the blue part of the flame of a Bunsen burner.

A.4 Staining of slides

A.4.1 Preparation of Soerensen-buffer

Dissolve 45,36 g KH₂PO₄ in 1 000 ml water (solution A) and dissolve 64,33 g Na₂HPO₄ in 1 000 ml water (solution B). Mix one part A, one part B and 8 parts of deionized water. This results in a solution with a pH of 6.8.

A.4.2 Staining

After storage for 2 d to 4 d in the dark, transfer the slides with the coated side up into dishes and cover with 1 cm of Soerensen-buffer. Thereafter, irradiate with UV-light (wavelength 254 nm) for 10 min from a distance of about 15 cm.

Transfer the slides into holders and immerse them in cuvettes which have previously been filled with “SSC twice” (a solution containing 0,3 mol/l NaCl and 0,03 mol/l of sodium citrate in deionized water). Incubate the slides in “SSC twice” for about 30 min at about 60 °C.

Thereafter, remove the slides and allow them to cool. Afterwards, place them for 10 min in their holders into cuvettes which had already been filled with a Giemsa solution, prepared by adding 15 ml of Giemsa to 185 ml Soerensen-buffer (pH 6.8).

Remove from staining and allow to dry. Remove the surplus of staining material in xylene and cover the slides. Protect slides against light.

A.5 Evaluation

Evaluate 200 metaphases per harvest time and note the numbers of the 1st, 2nd and 3rd metaphases.

The following criteria shall be met:

- in the 1st metaphases, both chromatids of all chromosomes are stained blue-violet;
- in the 2nd metaphases, one chromatid of all chromosomes is stained blue-violet, whereas the corresponding part of its sister chromatid is stained light-violet;
- in the 3rd metaphases, chromatids are stained predominantly light-violet.

A.6 Calculation of cell cycle length

Calculate the cell cycle length, l_{CC} , of the respective cell line according to Equation (A.1):

$$l_{CC} = \frac{t_h}{(1 \times n_1) + (2 \times n_2) + (3 \times n_3)} \times 100 \quad (A.1)$$

where

t_h is the harvest time in hours, h;

n_1 is the number of 1st mitosis in percent, %;

n_2 is the number of 2nd mitosis in percent, %;

n_3 is the number of 3rd mitosis in percent, %.

The mean of all four harvest times is used as mean cell cycle length for the respective cell line.

Annex B

(informative)

Evaluation schemes

B.1 Evaluation scheme for cell attachment and cell morphology

- 1 = good attachment and fibroblast-like cells
- 2 = slightly reduced attachment and some rounded cells
- 3 = reduced attachment and cells partly rounded and/or non-attached
- 4 = no attached cells, all cells rounded

B.2 Evaluation scheme for acceptance of preparations

- A = acceptable
 - 1 sufficient assessable cells of good quality
 - 2 reduced amount of assessable cells
 - 3 reduced amount of assessable cells, partly multi-fragmented nuclei
- B = limited acceptable
 - 1 severe reduction of assessable cells
 - 2 severe reduction of assessable cells, partly multi-fragmented nuclei
 - 3 interference of precipitate limiting slide reading
- N = not acceptable
 - 1 no or only few assessable cells
 - 2 severe interference of precipitate, no slide reading possible

Annex C (normative)

S9 fraction

C.1 Induction of liver enzymes

For enzyme induction, at least six male rats (e.g. Sprague-Dawley rats), each with about 200 g to 300 g of body mass, receive a single intraperitoneal injection of a polychlorinated biphenyl (e.g. Aroclor 1254) dissolved in an appropriate vehicle at a dose of 500 mg/kg body mass five days prior to termination. In the case of phenobarbital / β -naphthoflavone induction, the rats simultaneously receive 80 mg/kg body mass phenobarbital intraperitoneally and 80 mg/kg body mass β -naphthoflavone orally on three consecutive days in an appropriate vehicle. The livers are prepared 24 h after the last treatment.

Animals should be kept in special adequately marked cages which will be used only for this purpose. Animal husbandry should be standardized, with 12 h of electrical lighting daily, 20 °C to 23 °C room temperature, and about 60 % mean relative humidity, on a bedding of softwood granules. The air should be changed about ten times per hour. The animals should receive an appropriate standard diet and water of drinking quality ad libitum.

C.2 Preparation of the S9 fraction

Livers shall be removed under sterile conditions immediately after termination and kept at 4 °C \pm 1 °C until all the animals have been prepared. All the remaining steps should be carried out under sterile conditions at 4 °C \pm 1 °C.

Wash the livers with cold (4 °C \pm 1 °C), 0,15 mol/l KCl solution (approximately 1 ml KCl per 1 g liver). Homogenize the livers in fresh, cold (4 °C \pm 1 °C), 0,15 mol/l KCl (approximately 3 ml KCl per 1 g liver). Centrifuge the homogenate in a cooling centrifuge at 4 °C \pm 1 °C and 9 000 g for 10 min. Store the supernatant (the S9 fraction) below –70 °C in small portions.

10

Annex D (informative)

Precision data

The precision data given in Table D.1 were determined in an interlaboratory trial, organized by Germany in November 2005.

Table D.1 — Precision data

Sample No.	Sample	S9-Mix	<i>l</i>	Validity criteria		LID	Sample <i>o</i> %
				NC <i>o</i> %	PC <i>o</i> %		
1	Waste water No. 1 spiked with 1 µg/ml Mitomycin C	–	10	0	0	160	0
		+	10	10	0	20	0
2	Waste water No. 2	–	10	0	0	5	0
		+	10	0	10	5	10
3	Waste water No. 3 spiked with 4 µg/ml Cyclophosphamide	–	10	0	10	5	0
		+	9	0	0	20	20
4	Waste water No. 4	–	10	0	10	5	0
		+	10	0	0	5	0
Explanation							
<i>l</i>	is the number of laboratories;						
<i>o</i>	is the number of outliers;						
NC	is the number of negative controls;						
PC	is the number of positive controls;						
LID	is the lowest ineffective dilution (median).						

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