DRAFT INTERNATIONAL STANDARD

ISO/DIS 20184-3

ISO/TC **212**

Secretariat: **ANSI**

Voting begins on:
2020-08-03

Voting terminates on:
2020-10-26

Molecular *in vitro* diagnostic examinations — Specifications for pre-examination processes for frozen tissue —

Part 3:
Isolated DNA

Analyses de diagnostic moléculaire in vitro — Spécifications relatives aux processus préanalytiques pour les tissus congelés —

Partie 3: ADN extrait

ICS: 11.100.10

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ISO/CEN PARALLEL PROCESSING

Reference number
ISO/DIS 20184-3:2020(E)

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Published in Switzerland

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Foreword

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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).

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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 212, *Clinical laboratory testing and in vitro diagnostic test systems.*

A list of all parts in the ISO 20184 series can be found on the ISO website.

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.

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Introduction

Molecular in vitro diagnostics, including molecular pathology, has enabled significant progress in medicine. Further progress is expected with new technologies analysing nucleic acids, proteins, and metabolites in human tissues and body fluids. However, integrity of these molecules can change during specimen collection, transport, storage, and processing.

Consequently making the outcome from diagnostics or research unreliable or even impossible because the subsequent examination assay might not determine the real situation in the patient but an artificial profile generated during the pre-examination process. Therefore, a standardization of the entire process from specimen collection to DNA examination is needed. Studies have been undertaken to determine the important influencing factors. This document draws upon such work to codify and standardize the steps for frozen tissue with regard to DNA examination in what is referred to as the pre-examination phase.

DNA integrity in tissues can change during processing and storage. Modifications of the DNA molecules can impact the validity and reliability of the examination test results. Therefore, it is essential to take special measures to minimize the described DNA changes and modifications for subsequent examination.

In this document, the following verbal forms are used:

— “shall” indicates a requirement;

— “should” indicates a recommendation;

— “may” indicates a permission;

— “can” indicates a possibility or a capability.

Further details can be found in the ISO/IEC Directives, Part 2.

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Molecular *in vitro* diagnostic examinations — Specifications for pre-examination processes for frozen tissue —

Part 3: Isolated DNA

1 Scope

This document gives requirements and recommendations for the handling, storage, processing, and documentation of frozen tissue specimens intended for DNA examination during the pre-examination phase before a molecular examination is performed.

This document is applicable to molecular in vitro diagnostic examinations including laboratory developed tests performed by medical laboratories and molecular pathology laboratories that evaluate DNA isolated from frozen tissue. It is also intended to be used by laboratory customers, in vitro diagnostics developers and manufacturers, biobanks, institutions and commercial organizations performing biomedical research, and regulatory authorities.

Tissues that have undergone chemical stabilization pre-treatment before freezing are not covered in this document.

NOTE International, national, or regional regulations or requirements can also apply to specific topics covered in this document.

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 15189, *Medical laboratories — Requirements for quality and competence*

ISO 15190, *Medical laboratories — Requirements for safety*

ISO/IEC 17020, *Conformity assessment — Requirements for the operation of various types of bodies performing inspection*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 15189 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/

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3.1
aliquot
portion of a larger amount of homogenous material, assumed to be taken with negligible sampling error

Note 1 to entry: The term is usually applied to fluids. Solid tissues are heterogeneous and therefore cannot be aliquoted.

[SOURCE: Compendium of Chemical Terminology Gold Book. International Union of Pure and Applied Chemistry. Version 2.3.3., 2014; the PAC, 1990,62,1193 (Nomenclature for sampling in analytical chemistry (Recommendations 1990)) p. 1206; and the PAC 1990, 62, 2167 (Glossary of atmospheric chemistry terms (Recommendations 1990)) p. 2173.]

3.2
ambient temperature
unregulated temperature of the surrounding air

3.3
analyte
component represented in the name of a measurable quantity

[SOURCE: ISO 17511:2003, 3.2, modified — The example was not taken over. [16]]

3.4
analytical test performance
accuracy, precision, and sensitivity of a test to measure the *analyte* (3.3) of interest

Note 1 to entry: Other test performance characteristics such as robustness, repeatability can apply as well.

3.5
cold ischemia
condition after removal of the tissue from the body until its stabilization or fixation

[SOURCE: ISO 20184-2:2018, 3.5 [19]]

3.6
diagnosis
can involve *examinations* (3.9) and tests for classification of an individual's condition into separate and distinct categories or subclasses that allow medical decisions about treatment and prognosis to be made

[SOURCE: ISO 20184-2:2018, 3.6[19]]

3.7
DNA
deoxyribonucleic acid
polymer of deoxyribonucleotides occurring in a double-stranded (dsDNA) or single-stranded (ssDNA) form

[SOURCE: ISO 22174:2005, 3.1.2 [21]]

3.8
DNase
deoxyribonuclease
enzyme that catalyzes the degradation of *DNA* (3.7) into smaller components

[SOURCE: ISO 20184-1:2018, 3.8 [18]]

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3.9
examination
analytical test
set of operations with the object of determining the value or characteristics of a property

Note 1 to entry: Processes that start with the isolated *analyte* (3.3) and include all kinds of parameter testing or chemical manipulation for quantitative or qualitative examination.

[SOURCE: ISO 15189:2012, 3.7, modified — Notes to entry 1 to 3 have been removed. Note 1 to entry has been added and "analytical test" has been added as a preferred term.]

3.10
grossing
gross examination
inspection of pathology specimens with the bare eye to obtain diagnostic information, while being processed for further microscopic *examination* (3.9)

[SOURCE: ISO 20184-1:2018, 3.10 [18]]

3.11
homogeneous
uniform in structure and composition

[SOURCE: ISO 20184-1:2018, 3.11 [18]]

3.12
interfering substance
endogenous substance of a *specimen* (3.14)/*sample* (3.17) or exogenous substance (e.g. stabilization solution) that can alter an *examination* (3.9) result

[SOURCE: ISO 20184-1:2018, 3.12 [18]]

3.13
pre-examination process
preanalytical phase
preanalytical workflow
process that starts in chronological order, from the clinician's request and include the *examination* (3.9) request, preparation and identification of the patient, collection of the *primary sample(s)* (3.14), transportation to and within the medical or pathology laboratory, isolation of *analytes* (3.3), and ends when the analytical *examination* (3.9) begins

Note 1 to entry: The pre-examination phase includes preparative processes that influence the outcome of the intended *examination* (3.9).

[SOURCE: ISO 15189:2012, 3.15, modified — An additional term was added and more detail was included.]

3.14
primary sample
specimen
discrete portion of a body fluid, breath, hair or tissue taken for *examination* (3.9), study or analysis of one or more quantities or properties assumed to apply for the whole

[SOURCE: ISO 20184-1:2018, 3.14 [18]]

3.15
proficiency test
evaluation of participant performance against pre-established criteria by means of inter-laboratory comparisons

[SOURCE: ISO/IEC 17043:2010, 3.7, modified — The term and definition are used here without the original notes. [23]]

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3.16
room temperature
temperature in the range of 18 °C to 25 °C

Note 1 to entry: Local or national regulations can have different definitions.

[SOURCE: ISO 20184-1:2018, 3.19 [18]]

3.17
sample
one or more parts taken from a *primary sample* (3.14)

[SOURCE: ISO 15189:2012, 3.24, modified — The example was not taken over.]

3.18
stability
ability of a *sample* (3.17) material, when stored under specified conditions, to maintain a stated property value within specified limits for a specified period of time

Note 1 to entry: The *analyte* (3.3) for the purpose of this document is *DNA* (3.7).

[SOURCE: ISO Guide 30:2015, 2.1.15, modified — The words "reference material" were replaced by "sample material".[24]]

3.19
storage
maintenance of biological material under defined and standardized conditions for the intended use

Note 1 to entry: Long-term storage typically occurs in laboratory archives or in biobanks.

3.20
validation
confirmation, through the provision of objective evidence, that the requirements for a specific intended use or application have been fulfilled

Note 1 to entry: The term "validated" is used to designate the corresponding status.

[SOURCE: ISO 9000:2015, 3.8.13, modified — Note 1 and Note 3 were not taken over. [15]]

3.21
verification
confirmation, through provision of objective evidence, that specified requirements have been fulfilled

Note 1 to entry: The term "verified" is used to designate the corresponding status.

[SOURCE: ISO 9000:2015, 3.8.12, modified — Note 1 and Note 2 were not taken over. [15]]

Note 2 to entry: Confirmation can comprise activities such as:

— performing alternative calculations,

— comparing a new design specification with a similar proven design specification,

— undertaking tests and demonstrations, and

— reviewing documents prior to issue.

3.22
warm ischemia
condition before the tissue is removed from the body, but where it is deprived of its normal blood supply

[SOURCE: ISO 20184-1:2018, 3.25 [18]]

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3.23
workflow
series of activities necessary to complete a task

[SOURCE: ISO 20184-1:2018, 3.26 [18]]

4 General considerations

For general statements on medical laboratory quality management systems and in particular on specimen collection, reception and handling (including avoidance of cross contaminations) see ISO 15189, ISO/IEC 17025 or ISO/IEC 17020 [22]. The requirements on laboratory equipment, reagents, and consumables according to ISO 15189 shall be followed; ISO/IEC 17025 and ISO/IEC 17020 can also apply[22].

All steps of the pre-examination, examination and post-examination processes (i.e. the entire workflow) can influence the diagnosis or research study results. Thus, this entire workflow shall be specified, verified and validated during the development of the examination. This includes specifically all pre-examination process steps such as the examination request, preparation and identification of the patient, collection of the primary sample(s), transport to and within the medical laboratory, storage and isolation of analytes. Workflow steps which cannot always be controlled (e.g. warm ischemia) shall be documented.

In contrast to RNA or proteins, DNA in tissue is relatively stable during warm and cold ischemia. Changes of DNA sequence or copy numbers (e.g. comparative genomic hybridization (CGH) profiles) due to longer warm and cold ischemia durations are unknown[1]. However, DNA methylation patterns may change in response to ischemia[2]. The duration until the specimen is frozen should be kept as short as possible in order to avoid enzymatic degradation of DNA. The duration before freezing shall be documented and the temperature before freezing should be documented[1].

During the design and development of a DNA based examination, a risk assessment shall be performed (see also ISO 14971). Mitigation measures for eliminating or reducing identified risks shall be established where required for ensuring the performance of the examination.

Safety requirements on specimen transport and handling shall be considered (see ISO 15189 and ISO 15190). International, national, or regional regulations or requirements can also apply.

During the whole pre-examination process, precautions shall be taken to avoid cross contamination between different specimens/samples, e.g. by using single-use material whenever feasible or appropriate cleaning procedures between processing of different specimens/samples.

Where the examination manufacturer has specified instructions for pre-examination steps, these shall be followed. When, for justified reasons (e.g. unmet patient needs), a commercial product is not used in accordance to the manufacturer's instructions, responsibility for its validation, verification, use and performance lies with the user.

For general considerations on specimen collection, transport, receipt, and handling, see ISO 20658 and ISO 20387.

5 Outside the laboratory

5.1 Specimen collection

5.1.1 General

For the collection of the specimen, the requirements (e.g. disease condition, specimen size) for the intended molecular examination (see also Clause 6) should be considered.

See also ISO 15189.

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5.1.2 Information about the patient/specimen donor+

The documentation shall include the ID of the patient/specimen donor, which can be in the form of a code. The documentation should include, but is not limited to:

a) the relevant health status of the patient/specimen donor (e.g. healthy, disease type, concomitant disease and demographics (e.g. age and gender);

b) the relevant information about routine medical treatment and special treatment prior to tissue collection (e.g. anaesthetics, medications, surgical or diagnostic procedures);

c) the appropriate consent from the patient/specimen donor.

5.1.3 Information about the specimen

The documentation shall include, but is not limited to:

a) if required for the examination, the start of ischemia within the body (warm ischemia) by documentation of the ischemia-relevant vessel ligation/clamping time point (usually arterial clamping time);

NOTE Not needed where small tissue biopsy resection for freezing is performed.

b) the time and date when tissue is removed from the body and the method of removal (e.g. core-needle biopsy, resection, biopsy device used for the collection);

c) the description of tissue type and origin, tissue condition (e.g. diseased, unaffected by the disease), including references to any marking applied in or outside the operating theatre made by surgeon, radiologist or pathologist;

If the freezing of the tissue is performed outside the laboratory, the documentation steps described under 6.2, where pathology evaluation is required, and 6.3 shall be performed.

The documentation should also include the ID of the responsible person collecting the specimen, this can be in the form of a code.

5.1.4 Specimen processing

Tissues that need to be frozen for diagnostic purposes can originate from a large specimen or can be small specimens e.g. biopsies taken by endoscopy or taken from patients during a surgical procedure where fast frozen section diagnosis is required.

1) Any additions or modifications to the specimen after removal from the body (e.g. labelling for the orientation of the specimen (e.g. ink-marking, stitches, incision(s)) shall be documented.

Where a pathology diagnosis is required on the specimen, sampling shall be performed by or under supervision or guidance of a medically qualified (e.g. board certified) pathologist (see 6.2) and these personnel should be aware of all examinations for the specimen to ensure proper handling.

Where the specimen was removed without the requirement of pathology diagnosis, the evaluation, selection and documentation of specimens may be done by other qualified persons than pathologists.

2) Formalin fixation can have a negative impact on DNA based examinations (see ISO 20166-3) [17]. Frozen tissue samples are therefore preferred depending on the specific analytical examination. Where both DNA and RNA are intended to be examined, see ISO 20184-1 [18].

3) [18]. Freezing can be performed outside the laboratory or inside the laboratory under the direction or supervision of a pathologist.

Cold ischemia can influence the DNA pattern (e.g. fragmentation); therefore, immediate freezing according to 6.3.2 should be preferred.

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a) In case the specimen or sample is frozen outside the laboratory, proceed with 6.2 without delay.

b) In case the specimen or sample is frozen inside the laboratory, fresh tissue specimens need to be transported to the laboratory. The steps described under 5.2 for fresh tissue transport shall be performed without delay.

5.2 Fresh tissue transport requirements

5.2.1 General

Where transport of the specimen or sample to the laboratory is required for freezing, the laboratory in partnership with the clinical or surgery department shall provide instructions for the transport procedure of the specimen.

5.2.2 Preparations for the transport

The following steps shall be performed:

a) the selection and use of containers and packages (e.g. cooling box, box for storing and transportation, vacuum packaging);

NOTE 1 This will be in accordance with applicable transport regulations.

b) the selection and use of stabilization procedures (e.g. cooling methods) for transport;

NOTE 2 Accidentally freezing of the fresh tissue (e.g. by using cool packs incorrectly) can lead to DNA degradation when the tissue thaws. It can also impact the morphological characterization.

c) the labelling of the container (e.g. registration-number, barcode, specimen type, quantity, and organ tissue of origin) and additional documentation (information as specified in 5.1.2, 5.1.3, 5.1.4, and 5.2.2, a) to b)).

A single container may only contain several specimens, if these specimens come from the same location (i.e. similar or corresponding anatomical or histological organ parts) and have the same macroscopic features such as tissue type and disease status (e.g. inflammation, tumour and necrosis).

NOTE 3 Specimens that have the same macroscopic features can have different molecular features.

After removal from the body, specimens should be transferred without delay into the container. The container should then be kept on wet-ice or at 2 °C to 8 °C in order to minimize changes to the DNA (e.g. fragmentation). Alternatively, short exposure (e.g. up to 3 hours) to room temperature may be acceptable depending on the anticipated examination requirements. During the development of the examination, the maximum storage duration at defined temperature ranges shall be specified and verified.

The temperatures of the collection container's surroundings during cold ischemia (e.g. temperatures in different rooms, transport) should be documented. If the temperature is not measured, the temperature range should be estimated by classification as ambient temperature, room temperature, or at 2 °C to 8 °C.

5.2.3 During transport

Temperature monitoring should be applied in a suitable manner (e.g. temperature tracking). If the temperature cannot be measured, the temperature should be estimated and documented (e.g. as ambient temperature, room temperature, at 2 °C to 8 °C or wet ice (pack)).

If the specimen is not already frozen, it should be transported on wet ice (pack) or at 2 °C to 8 °C without delay in order to minimize changes to the DNA.

The compliance with the protocol for the transport procedure shall be documented. Any deviations from the protocol shall be described and documented.

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6 Inside the laboratory

6.1 Information about the reception of the specimen

The name of the person receiving the specimen, which can be in the form of a code, shall be documented. The specimen arrival date and time and conditions (e.g. labelling, transport conditions including temperature, tissue type and number of specimens, leaking/breaking of the container) of the received specimens shall be documented. Any deviations from the established protocol for the transport procedure (see 5.2) shall be documented.

The correct identity of the specimen shall be checked. This should include the clinical information (see 5.1.2 and 5.1.3) of the specimen (e.g. type, size, number), hospital admission number and/or donor/patient ID, name of the patient, date of birth of the patient and type of tissue. Non-conformities to instructions, e.g. labelling, storage and transport conditions (temperature, and duration), specimen and leaking/broken collection devices, shall be documented.

6.2 Evaluation of the pathology of the specimen and selection of the sample(s)

The evaluation and documentation of the pathology of the specimen and the selection of the sample(s) from the specimen for further processing shall be done by or under supervision or responsibility of a medically qualified (e.g. board certified) pathologist.

Local, national or regional regulations may apply.

Options to select the sample(s) for DNA examination:

a) The selection of appropriate parts of the specimen for molecular and histopathological examinations as well as for optional further research purposes shall be done by or under supervision of a medically qualified (e.g. board certified) pathologist to ensure that the collection of the sample for DNA examination does not compromise the histopathological analyses.

   For molecular examination, suitable tissue parts should be selected, whereas parts potentially compromising the molecular examination, such as bleeding and necrotic parts, should be avoided where appropriate. Micro- or macro- dissection of tissue should be considered to select or enrich for certain cellular features of a disease.

   NOTE 1 Depending on local procedures, the selection of appropriate parts of the specimen can also be done outside of the laboratory, e.g. in the operating theatre (see 5.1.4).

   In the context of macroscopic evaluation of the surgical specimen the correct identity of the specimen shall be checked before and/or after freezing. The clinical information (see 5.1.2 and 5.1.3) of the specimen (e.g. type, size, number), hospital admission number and/or pathology case number and/or donor/patient ID, name of the patient, date of birth of the patient and type of tissue should be checked. The surgical specimen and all findings shall be described appropriately according to the guidelines of the respective medical societies and in correlation with the clinical information and questions. The anatomic localization of the specimen shall be described, resection margins and other important areas may be marked if necessary and helpful for later microscopic evaluation; photographs may be taken. Representative samples for microscopic evaluation shall be taken (i.e. grossing) according to the organ/disease specific guidelines from the respective medical societies.

   NOTE 2 The above described evaluation or documentation can also be done outside of the laboratory, e.g. in the operating theatre.

b) When the specimen was removed from the body without the requirement of histopathological diagnosis, documentation of this specimen, the evaluation, selection and documentation of the samples may be done by other qualified persons than pathologists.

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c) When frozen section diagnosis is required, the selected part of the specimen shall be frozen (see 6.3) in an appropriate freezing medium. The freezing medium used shall be documented. Frozen sections shall be evaluated by a medically qualified (e.g. board certified) pathologist.

6.3 Freezing of the specimen or sample(s)

Freezing of the specimen or sample(s) shall be performed without delay. The most optimal freezing method (see 6.3.1) for the specimen or sample in regard to the intended use and the working conditions should be chosen and used.

The vial shall be labelled before it is pre-cooled.

The three possible freezing procedures are as follows:

1) Snap-freezing procedure[3]: This method should be preferred as it gives the best preservation of morphology in frozen tissue samples[4]. Isopentane (C5H12, also called methylbutane or 2-methylbutane), shall be pre-cooled ranging from ≤ −80 °C to > −160 °C where it can be used for snap-freezing. Pre-cooling can be done with liquid nitrogen (−196 °C), dry ice (−80 °C), −80 °C freezers or dedicated freezing appliances that keep the isopentane ≤ −80 °C with or without controlled cooling rate. The isopentane shall be cooled in a tube or other container (e.g. glass beaker) resistant to the large and sudden temperature shifts. The volume of pre-cooled isopentane shall be at least 10x the volume of the specimen or sample. For snap-freezing the tissue sample shall be completely submerged into the pre-cooled isopentane.

   After the tissue is frozen, it shall be transferred into its designated pre-cooled labelled cryo-vial. The vial shall be closed according to the manufacturer's instructions. The isopentane should be refreshed when debris is seen at the bottom of the tube or container that contains the isopentane used for snap-freezing.

   Isopentane is an extremely volatile and extremely flammable liquid at room temperature and pressure. Therefore, the laboratory should be well ventilated. The isopentane in the tube should be cooled.

2) Fast freezing procedure: Tissues shall be fast frozen on a pre-cooled metal plate, or metal basket placed on the surface of liquid nitrogen, or on dry ice. The metal surface shall be pre-cooled ranging from ≤ −80 °C to > −196 °C. The metal plate or metal basket can be fixed into position with a suitable stand and clamp. Alternatively, the sample can be frozen directly in liquid nitrogen or even in the labelled and closed storage vial in liquid nitrogen or in dry ice. However, a slow freezing process can cause membrane disruption by compartmental rising salt concentrations and crystal formation which can seriously affect the morphology[5]. To avoid cross-contamination, the basket or plate should be cleaned between freezing samples.

   NOTE 1 Freezing in liquid nitrogen is characterized by the Leidenfrost effect [6] caused by boiling of liquid nitrogen around the tissue due to its relatively high temperature. This reduces the heat conduct from the sample to the liquid nitrogen; this becomes worse when the sample is placed in the vial before freezing.

3) Procedure for freezing tissue for fast frozen section diagnosis (see also 6.2c): The tissue should be transported freshly to the laboratory without delay. The selected part of the specimen shall be frozen onto the specialized support device (e.g. metal grid, pedestal, disc) that fit onto the cryostat in an appropriate freezing medium. The freezing medium used shall be documented. The support device containing the tissue and freezing medium should be frozen preferably in liquid nitrogen or dry ice for improved morphology. After cutting the frozen sections, the remainder should be removed from the support device without thawing and stored in a pre-cooled vial for long term storage.

   Samples treated with freezing medium should be stored at ≤ −70 °C to avoid dryness and the DNA quality should be evaluated for each freezing medium treated sample before use (see 6.6).

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The following steps shall be performed before, during and after the freezing procedure:

a) the documentation of the freezing procedure (e.g. freezing in liquid nitrogen, snap-freezing in isopentane cooled by liquid nitrogen or dry ice, freezing in an appropriate freezing medium, freezing with controlled cooling rate);

b) the documentation of the freezing time point and date (to determine the lag time: time period between removal from the body – until freezing of the specimen or sample);

c) the selection of the cryo-vial for cryo-storage:

   1) the cryo-vial shall have a sufficient volume for the size of the specimen or sample to be stored in;

   2) the cryo-vial shall be certified for the storage temperature;

   3) the cryo-vial shall be safely closable, preferably with screw caps; containers with a flip cap shall not be used;

      NOTE 2 Due to incorrect closure, containers can explode upon specimen or sample retrieval when they have been stored under liquid nitrogen and leakage of liquid nitrogen in the tube has occurred.

d) determine if the required tissue size of the sample fits into the chosen cryo-vial before freezing, because the tissue size determines the size of the container; it is therefore recommended, that the specimen or sample does not exceed 1 cm in one dimension;

e) the labelling shall be suitable for the respective frozen storage conditions;

   NOTE 3 Suitable labels are e.g. self-adhesive labels, handwriting, radio frequency identification (RFID), pre-labelled containers, which have been verified for purpose.

f) the labelling of the container shall ensure appropriate traceability of specimens and samples. Therefore, the container labelling shall provide the minimum information of:

   1) the ID of the patient/specimen donor, unique specimen/sample ID and date when the specimen and/or sample was collected, which all can be in the form of a code (unique for every specimen/sample);

   2) the basic information on e.g. the tissue type, tissue and disease condition such as affected (e.g. tumour) and/or unaffected, unless a sample tracking system can supply this information coupled to the identification of the specimen or sample used in 6.3 f) 1);

   3) the unique numbering of each cryo-vial, which can be included in 6.3 f) 1);

g) the documentation of types, quantity and description of the specimen(s) or sample(s).

It should be considered that under some disease conditions, such as tumours, molecular features may not be present homogeneously in the tissue sample. Therefore, it is important that the part of the actual tissue sample used for molecular examination is evaluated by a medically qualified (e.g. board certified) pathologist. In this context it should be documented which aspect of a disease is actually reflected in the tissue sample used for molecular examination (e.g. different molecular mechanisms can be activated at the centre or the invasion front of the tumour, also tumours can be composed of areas showing variations in differentiation grades).

6.4 Storage requirements

The constant temperature shall be ≤ –70 °C. Systems monitoring the temperature should be used.

Freezers or liquid nitrogen tanks shall have a temperature alarm system.

Major temperature shifts can occur during retrieval of the specimen(s) or sample(s). Therefore, retrieval times should be kept as short as possible to avoid the thawing of samples.

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Temperature shifts occurring, that can have accidentally thawed the specimen(s) or sample(s) to be processed or to be further stored, shall be documented.

Back-up cryo-storage facilities should be provided.

The storage position, storage temperature, time and date of the retrieval of any specimen or sample from the storage system shall be documented.

6.5 DNA isolation

6.5.1 General

A histopathological characterization of the cellular composition and disease condition of the specimen or sample shall be performed (e.g. on hematoxylin/eosin (H&E) sections) according to an internationally defined histopathological classification (e.g. [7]). When the specimen or sample is used for molecular in vitro diagnosis, the fraction of target cells shall be determined based on the examination requirements prior to the DNA isolation. The quantity of target cells shall be sufficient to perform the examination. When the specimen or sample is not used for molecular in vitro diagnosis, e.g. for research, a similar approach is recommended.

1) All materials that can contact the sample or tissue slides, that includes the lysis buffer and vial containing this buffer, vials and tools used to manipulate the frozen sample for cryo-sectioning or transferring to the lysis buffer, shall be clean and nuclease free. All materials (excluding the lysis buffer and vial containing this buffer) and tools used shall be cooled to < 0 °C before manipulating the frozen sample for cryo-sectioning and transferring to the lysis buffer . The person handling the material(s) and/or frozen sample shall wear gloves. The relevant parts of the microtome, including the reusable blade, shall be cleaned after the cutting of each frozen tissue specimen/sample. The use of new disposable blades on the microtome should be considered to avoid cross-contaminations.

2) Where morphology changes (e.g. tumour content), it can influence the examination results; cryo-sections should be used for DNA isolation where the morphology is checked after every 50 µm by cutting a section for Hematoxylin and Eosin (H&E) staining.

3) The method used as well as kits and lot numbers used in the process shall be documented.

The isolated DNA should be kept on wet-ice or at 2 °C to 8 °C (e.g. cooling block) and should be assayed or put into the long-term storage (6.7) immediately.

To avoid a cross contamination with amplified material from the DNA examination, the isolation of the DNA should not be performed in the same area as the amplification steps of the examination process, unless a closed system is used, which is designed to avoid cross-contamination.

If there is doubt in the correct identification of the specimen or sample, the identity of the specimen/sample shall be verified.

The isolation of DNA is a key step in the diagnostic workflow, which shall be especially focused on during the validation of the entire workflow.

The DNA isolation method should be tested in a DNA proficiency test program.

6.5.2 Using commercial kits

When using commercial kits dedicated to the isolation of DNA from frozen tissues, the manufacturers' instructions for use shall be followed.

6.5.3 Using laboratory developed protocols

If a commercial kit is not used in accordance to its intended use but is validated fit for purpose as defined by the user, instructions shall be written and followed.

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If the laboratory uses its own protocol independently from a commercial kit, the validation demonstrating that it is fit for purpose shall be done, and instructions shall be written and followed.

The use of products from different manufacturers can compromise results as the products may not be compatible. They should be used for diagnostic testing only if the components have been tested together and validated to work satisfactorily.

A laboratory developed DNA isolation procedure for frozen tissue sections should contain the following steps:

1) Resuspension of tissue sections in a lysis buffer;

2) Digestion with proteinase K to remove proteins and to release the DNA from larger sections or fragments. For the proteinase K digestion step at e.g. 55 °C to 56 °C, the lysis buffer should be optimized followed by a proteinase K heat inactivating step at e.g. 95 °C.

   NOTE 1 Some commercial kits and laboratory developed proteinase K protocols are available for sequential RNA then DNA isolation from the same tissue. Such protocols often result in some loss of DNA into the RNA extraction and may impact the expected DNA yield from a set amount of tissue.

3) Optional: Incubation with RNase A in case RNA-free genomic DNA is required.

   NOTE 2 When using a laboratory developed method, too high amounts of RNA can be co-purified with the DNA, which can disturb the intended examination. In such cases, it can be required to incorporate an RNase digestion step.

   NOTE 3 Co-purified RNA, even after digestion, can lead to over quantification of DNA yield by spectrophotometric methods, if digested RNA fragments are not sufficiently removed during the DNA purification procedure. This over quantification and the actual presence of RNA can also interfere with the examination test.

4) Isolation of the DNA from the lysate, e.g. by phenol/chloroform-based methods or by using commercially available kits suitable for DNA purification from frozen tissue.

6.6 Quantity and quality assessment of isolated DNA

The DNA quantity and quality should be checked according to the diagnostic kit manufacturer's instructions, or according to validated procedures by generally accepted physical, chemical and biochemical procedures. ISO 20395 provides more specific guidance for DNA quality and quantity measurement validation [20]. Procedures may include one or more of the following techniques, depending on the specific examination test:

a) quantification by absorbance measurements (A260), spectrofluorometry or a genome specific qPCR assay [8], [9];

b) test for purity by absorbance measurements (e.g. wavelength scan, A260/A280 ratio to assess protein contamination; A260/A230 ratio to assess contamination with phenol, salts, alcohol, etc.);

c) test for DNA integrity and amplifiability (by e.g. electrophoresis, chromatography, or molecular methods such as the differential length amplicon ratio) [10], [11];

d) test for presence of interfering substances (using exogenous controls (spiked in DNA controls) or inspecting qPCR response curves for anomalies) [12].

NOTE For qualitative examinations, such as presence/absence, sequencing, copy number variation 6.6, a) and b) are often applied; for quantitative examinations 6.6, a) to d) can be required.

6.7 Storage of isolated DNA

For storing the isolated DNA, the examination manufacturer's instructions shall be followed. Where these are not available, the DNA isolation kit manufacturer's instructions shall be followed.

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Where none of the information above is available, the laboratory shall use its own specified and verified procedure for storing isolated DNA.

If there is no information available from the DNA isolation kit provider, or if the laboratory's own validated DNA isolation procedures are used, the laboratory shall have specified and verified procedures in place on how to store the isolated DNA.

For long term storage the DNA should be eluted in weakly alkaline elution buffer, e.g. TE buffer which is a 10 mM Tris solution including 1 mM EDTA and brought to pH 8,0 with HCl for DNA examinations.

NOTE Depending on the DNA isolation procedure and the resulting eluate quality, storage at room temperature for a short period of time or at 2 °C to 8 °C can be appropriate in certain circumstances.

Storage for long-term purposes should be at –20 °C or below. Other validated methods for archiving can also be used [13], [14].

Appropriate storage vessels, such as cryo-vials, should be used.

In general, multiple freeze-thaw cycles should be avoided. For long-term storage, aliquots of the isolated DNA should be generated. Any thawing and refreezing of a DNA aliquot shall be documented.

For long-term storage, a validated process should be in place to organize and uniquely mark the storage vessel containing the isolated DNA or aliquots derived therefrom.

Traceability shall be ensured, e.g. by the use of readable RFID, 1D- or 2D-barcodes or pre-printed storage vessels with unique codes provided by manufacturers suitable for low storage temperatures.

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