INTERNATIONAL STANDARD

ISO 20184-1

First edition
2018-11

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

Part 1: Isolated RNA

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

Partie 1: ARN extrait

Reference number
ISO 20184-1:2018(E)

ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Fax: +41 22 749 09 47
Email: copyright@iso.org
Website: www.iso.org

Published in Switzerland

Contents

Page

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

A list of all parts in the ISO 20184 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.

Introduction

Molecular in vitro diagnostics, including molecular pathology, has enabled a significant progress in medicine. Further progress is expected with new technologies analysing nucleic acids, proteins, and metabolites in human tissues and body fluids. However, the profiles and/or integrity of these molecules can change drastically during specimen collection, transport, storage, and processing thus making the outcome from diagnostics or research unreliable or even impossible because the subsequent examination assay will not determine the 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 the RNA 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 RNA examination in what is referred to as the pre-examination phase.

RNA profiles in tissues can change drastically before, during and after collection (due to e.g. gene induction or gene down regulation). RNA species can change differently in different donor's patients' tissues.

Therefore, it is essential to take special measures to minimize the described RNA profile changes and modifications within the tissue 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.

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

Part 1: Isolated RNA

1 Scope

This document gives guidelines on the handling, documentation, storage and processing of frozen tissue specimens intended for RNA examination during the pre-examination phase before a molecular assay is performed.

This document is applicable to any molecular in vitro diagnostic examination performed by medical laboratories and molecular pathology laboratories that evaluate RNA extracted from frozen tissue. It is also intended to be used by laboratory customers, in vitro diagnostics developers and manufacturers, biobanks, institutions and commercial organisations 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 15189:2012, *Medical laboratories — Requirements for quality and competence*

3 Terms and definitions

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

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. Tissues are heterogeneous and therefore cannot be aliquoted.

Note 2 to entry: The definition is derived from References [22], [23] and [24].

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]

3.4
analytical test performance
accuracy, precision, and sensitivity of a test to measure the analyte 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 stabilization or fixation

3.6
diagnosis
identification of a health or disease state from its signs and/or symptoms, where the diagnostic process can involve examinations 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

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]

3.8
DNase
deoxyribonuclease
enzyme that catalyzes the degradation of DNA into smaller components

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

Note 1 to entry: Processes that start with the isolated analyte 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

3.11
homogeneous
uniform in structure and composition

3.12
interfering substances
endogenous substances of a specimen/sample or exogenous substances (e.g. stabilization solution) that can alter an examination result

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

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

[SOURCE: ISO 15189:2012, 3.15, modified — "pre-analytical workflow" has been added as a preferred term, Note 1 to entry has been added and the definition has been extended.]

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

[SOURCE: ISO 15189:2012, 3.16, modified — Notes to entry 1 to 3 have been removed.]

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

[SOURCE: ISO 17043:2010, 3.7, modified — Notes to entry 1 and 2 have been removed.]

3.16
RNA profile
amounts of the individual RNA molecules that are present in a sample and that can be measured in the absence of any losses, inhibition and interference

3.17
RNA
ribonucleic acid
polymer of ribonucleotides occurring in a double-stranded or single-stranded form

[SOURCE: ISO 22174:2005, 3.1.3]

3.18
RNase
ribonuclease
enzyme that catalyses the degradation of RNA into smaller components

3.19
room temperature
for the purposes of this document, temperature in the range of 18 °C to 25 °C

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

3.20
sample
one or more parts taken from a primary sample

[SOURCE: ISO 15189:2012, 3.24, modified — EXAMPLE has been removed.]

3.21
stability
characteristic of a sample material, when stored under specified conditions, to maintain a specified property value within specified limits for a specified period of time

Note 1 to entry: The analyte for the purpose of this document is isolated RNA.

[SOURCE: ISO Guide 30:2015, 2.1.15, modified — "reference material" has been replaced by "sample material", "characteristic" has been replaced by "ability" and Note 1 to entry has been changed.]

3.22
storage
prolonged interruption of the pre-analytical workflow of a sample or analyte respectively, or of their derivatives e.g., stained sections or tissue blocks, under appropriate conditions in order to preserve their properties

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

3.23
validation
confirmation, throughout 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 — Notes to entry 1 and 3 have been removed.]

3.24
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 where not taken over.]

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;

— reviewing documents prior to issue.

3.25
warm ischemia
condition before the tissue is removed from the body, but where it is deprived of its normal blood supply

3.26
workflow
series of activities necessary to complete a task

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 15189:2012, 4.2, 5.4.4, 5.4.6 or ISO/IEC 17020:2012, Clause 8 and 7.2. The requirements on laboratory equipment, reagents, and consumables in accordance with ISO 15189:2012, 5.3 shall be followed; ISO 15189:2012, 5.5.1.2 and 5.5.1.3, and ISO/IEC 17020:2012, 6.2 can also apply.

All steps of a diagnostic workflow can influence the final analytical test result. Thus, the entire workflow including biomolecule stability and sample storage conditions shall be verified and validated. Workflow steps which cannot always be controlled (e.g. warm ischemia) shall be documented. A risk assessment of non-controllable workflow steps including their potential impact on the analytical test performance shall be performed and mitigation measures shall be established to enable the required analytical test performance.

Before or during the design of an analytical test, it should therefore be investigated and assured that the RNA profile(s) intended to be analysed is/are not compromised in a manner impacting the analytical test performance (e.g. by performing a time course experiment or study; see also Annex A).

Before tissues are stabilized by freezing, the RNA profile(s) can change e.g. by gene induction, gene down regulation and RNA degradation. These effects depend on the warm and cold ischemia duration and the ambient temperature before freezing. In addition, the described effects can vary in different donors'/patients' tissues.

Generally, the longer the duration of warm and cold ischemia and the higher the ambient temperature before freezing the tissue specimen, the higher is the risk that changes in the RNA profile can occur.

NOTE Intraoperative warm ischemia can result in more pronounced changes of RNA profiles than during postoperative cold ischemia[7][8]. RNA profiles can also vary, depending on the origin and type of tissue, the underlying disease, the surgical procedure, the drug regimen, and drugs administered for anaesthesia or treatment of concomitant disease and on the different environmental conditions after the tissue removal from the body.

As warm ischemia cannot be easily standardized, its duration shall be documented. When it is not possible to avoid cold ischemia, duration shall be documented and temperatures of the specimen container's surroundings should be documented. Where the specimen is transported to another facility for freezing, the transport duration shall be documented and the ambient conditions should also be documented.

Safety instructions on transport and handling shall be considered (see ISO 15189:2012, 5.2.3 and 5.4.5 and ISO 15190).

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.

If a commercial product is not used in accordance with the manufacturer's instructions, responsibility for its use and performance lies with the user.

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 15189:2012, 5.4.4.

5.1.2 Information about the specimen donor/patient

The documentation shall include the ID of the specimen donor/patient, 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 specimen donor/patient (e.g. healthy, disease type, concomitant disease, demographics [e.g. age and gender]);

b) the 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 specimen donor/patient.

5.1.3 Information about the specimen

The documentation shall include, but is not limited to:

a) 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 of steps described under 6.2, where pathology evaluation is required, and 6.3 has to be performed.

The documentation should also include the ID of the responsible person collecting the specimen.

5.1.4 Specimen processing

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

Post-mortem tissues can be frozen for diagnostic purposes. However, preservation of RNA is dependent on the time interval between death and autopsy and the temperature of storage of the body after death.

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).

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.

The freezing of the specimen or samples taken from the specimen can be performed outside the laboratory or inside the laboratory.

Cold ischemia can influence the RNA profile; therefore direct freezing should be preferred.

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 collaboration with the clinical or surgery department shall establish a protocol 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) according to applicable transport regulations;

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

NOTE Accidentally freezing the tissue (e.g. by using cool packs in a wrong manner) will lead to RNA 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, and 5.1.4 and 5.2.2 a) and b)].

Several specimens from the same patient/donor sharing similar features (macroscopic appearance, tissue type, disease status, anatomical location) may be put into a single container/container compartment.

Specimens should be transferred without delay into the container after the removal from the body. The container should then be kept on wet-ice or at 2 °C to 8 °C in order to minimize RNA profile changes.

The temperatures of the 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.

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

NOTE There is evidence that RNA in tissues can be stabilized in plastic bags under vacuum when kept at 0 °C to 4 C° during transport before the samples are archived for biobanks or used for histopathological evaluation[9].

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

6 Inside the laboratory

6.1 Information about the reception of the specimen

The name of the person receiving the specimen shall be documented. The specimen arrival date and time, and conditions (e.g. labelling, transport conditions including temperature, tissue type and quantity of the specimen, 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.

NOTE Temperature conditions during transport can influence the RNA profile and RNA quality.

The correct identity of the specimen shall be checked. This should include the clinical information (see 5.1.1 and 5.1.3) of the specimen, hospital admission number and/or donor/patient ID, name of the patient, date of birth of the patient.

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 can apply.

Options to select the sample(s) for RNA 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 RNA 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. Microdissection 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 the macroscopic evaluation of the surgical specimen 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 represented in 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 tissue specimen was removed from the body without the requirement of a histopathological diagnosis, documentation of this specimen as well as the evaluation, selection and documentation of the samples may be done by other qualified persons than pathologists.

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 6.3.2) for the specimen or sample in regards 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.

a) Snap-freezing procedure[10]: This method should be preferred as it gives the best preservation of morphology in frozen tissue samples. Isopentane (C_5H_{12}, 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 10 times 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 tissue sediment is seen at the bottom of the tube.

   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.

b) 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. To avoid cross-contamination, the basket or plate should be cleaned between freezing samples.

   NOTE Freezing in liquid nitrogen is characterized by the Leidenfrost effect[11] 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 labelled vial.

c) Frozen section procedure: For freezing tissue for fast frozen section diagnosis: The tissue should be transported freshly to the laboratory without delay. The selected part of the specimen shall be frozen onto the specialized metal grids that fit onto the cryostat in an appropriate freezing medium. The freezing medium used should be documented. The metal grid containing the tissue and freezing medium is frozen by holding the metal in liquid nitrogen or dry ice until the tissue is frozen. After cutting the frozen sections the remainder should be removed from the metal grid without thawing and stored in a pre-cooled vial for long term storage.

NOTE 1 The use of a freezing medium is known to harm protein mass spectrometry where the column separation can become impaired.

NOTE 2 Samples treated with freezing medium should be stored at −70 °C to avoid dryness and the RNA quality should be evaluated for each freezing medium treated sample before use.

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) 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/sample does not exceed 1 cm in one dimension;

d) the selection of the specimen/sample container for cryo-storage:

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

   2) the container shall be certified for the storage temperature;

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

      NOTE Containers can explode upon specimen or sample retrieval when they have been stored in liquid nitrogen.

   4) the containers shall be suited for permanent labelling under frozen storage conditions;

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

   NOTE 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 specimen donor/patient, 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) or unaffected, unless a sample tracking system can supply this information coupled to the identification of the specimen or sample used in 6.3.3 f) 1).;

   3) the unique numbering of each container, which can be included in 6.3.3 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 and controlling the temperature should be implemented and 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.

Occurring temperature shifts, 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 Isolation of RNA

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. WHO/IARC Classification of Tumours[12]). When the specimen or sample is used for molecular diagnosis, the fraction of target cells shall be evaluated prior to the RNA isolation. The quantity of target cells shall be sufficient to perform the examination. When the specimen or sample is not used for diagnosis, e.g. for research, a similar approach is recommended.

Freezing of tissue may result in the disruption of cellular membranes and organelles within the tissue. As a consequence, after thawing, enzymes may be released and activated which may lead to degradation of RNA. Therefore, the specimen or sample shall not thaw before its homogenous dispersion in lysis buffers containing RNase inhibiting substances. The specimen or sample should be thoroughly minced or cut into small pieces in its frozen state and thoroughly dispersed with lysis buffers containing RNase inhibiting substances. The subsequent homogenization of the frozen specimen or sample in the lysis buffer shall be processed immediately after having introduced the tissue into the lysis buffer. This step has a major impact on the stabilization of the RNA integrity and yield and should be controlled in the laboratory. Suitable homogenization devices for isolation of RNA from tissues are commercially available.

Alternatively, sections that have been cut on a cryotome (recommended thickness of 4 µm to 20 µm) shall be submerged, while still frozen, directly into the lysis buffers containing RNase inhibiting substances.

In case, the processed specimen or sample contains freezing medium, this shall be documented.

6.5.2 Requirements and recommendations

a) 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 nuclease free. All materials (excluding the lysis buffer and vial containing this buffer) and tools used to manipulate the frozen sample for cryo-sectioning or transferring to the lysis buffer shall be cooled to <0 °C while kept in an environment of ≤–20 °C before use. Histotechnologists 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.

b) Where morphology changes (e.g. tumour content) it can influence the examination results, it is strongly recommended to use cryo-sections for RNA isolation and check the morphology after every 50 µm by cutting a section for Hematoxylin and Eosin (H&E) staining.

c) The incorporation of a DNase treatment step into the RNA isolation procedure is recommended. The DNase, other reagents and consumables coming in touch with the RNA shall be RNase-free.

d) The method used as well as kits and lot numbers used in the process need to be documented.

e) The extracted RNA should be kept on wet-ice or at 2 °C to 8 °C (e.g. cooling block) and should be assayed immediately.

f) To avoid a cross contamination with amplified material from the RNA examination, the isolation of the RNA 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, an identification verification test shall be performed.

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

The RNA isolation performance should be tested in a RNA proficiency test program.

6.5.3 Using commercial kits

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

6.5.4 Using the laboratories own 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.

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.

6.6 Quantity and quality assessment of isolated RNA

The RNA quantity and quality should be checked according to the diagnostic kit manufacturer's instructions, or where provider's instructions are not available, by generally accepted physical, chemical and biochemical procedures[13][14][15]. These may include one or more of the following, depending on the specific examination:

a) quantification by absorbance measurements (A_{260}) or spectrofluorometry;

b) test for purity by absorbance measurements (e.g. wavelength scan, A_{260}/A_{280} ratio);

c) test for RNA integrity (by e.g. electrophoresis, chromatography, molecular methods such as the 3′/5′ assay or differential length amplicon ratio[16][17][18], or microfluidic methods to determine quality coefficients [e.g. RNA Integrity Number (RIN), RNA Quality Indicator (RQI)]);

d) test for presence of interfering substances (using exogenous controls (spiked in RNA and DNA controls) or inspecting qPCR response curves for anomalies)[19][20] or using an endogenous RNA for an RT-PCR inhibition test by introducing increasing eluate volumes into the examination.

NOTE For qualitative examinations, such as presence/absence of a RNA profile , 6.6, a)and b) can be sufficient; for quantitative examinations, such as gene expression, examination 6.6, a) to d) can be required.

6.7 Storage of isolated RNA

6.7.1 General

For long-term storage, usually the isolated RNA is frozen. However, for RNA preservation other validated methods for archiving can also be used.

For long-term storage, aliquots of the isolated RNA should be generated to avoid repeated freezing and thawing or repeated recovery from other archiving systems. The aliquots should not be further diluted to avoid a reduction of the RNA quality.

For small RNA amounts, storage vessels with reduced nucleic acid adsorption to the tube wall should be used.

Unintended freeze-drying of the isolated RNA during long-term storage due to water evaporation should be avoided as the RNA can degrade, and the recovery from the storage vessel can be difficult or even impossible. Therefore, appropriate storage vessels, such as cryogenic vials to avoid water evaporation during long-term storage, should be used. The storage vessel type and cap type should be documented.

For long-term storage, a validated process should be in place to organize and uniquely mark the storage vessel containing the isolated RNA 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.

6.7.2 Using commercially available kits for RNA isolation

The RNA isolation kit provider's specific instructions for storing the isolated RNA before the examination, should be followed. Where the examination provider’s instructions are more stringent, these shall be followed.

6.7.3 Using the laboratory's own protocols for RNA isolation

If there are neither instructions available from the RNA isolation kit provider nor from the examination provider, or if the laboratory's own validated RNA isolation procedures are used, the isolated cellular RNA should be assayed immediately, the laboratory shall have verified procedures [including appropriate storage medium (e.g. RNase-free water)] in place on how to store the isolated RNA before the analytical phase.

NOTE 1 Depending on the RNA isolation procedure the resulting RNA eluate quality, storage on wet-ice for a short period of time (i.e. 1 h) can be appropriate in certain circumstances.

For long-term storage, isolated RNA should be eluted in an appropriate buffer and stored at ≤–70 °C. Other validated methods for archiving can also be used[21].

NOTE 2 Some RNA isolation procedures can allow storing the RNA in the range from –20 °C to –70 °C.

Annex A
(informative)

Impact of pre-examination variables on RNA profiles obtained from frozen liver tissue samples collected during and after routine surgery

A.1 Comparison of stable and unstable genes identified under ischaemic conditions

Within the EU FP7 SPIDIA project[1)] a comprehensive multicentre study was performed to identify changes of RNA profiles by pre-examination variation in tissue samples. Non-malignant tissue samples were collected at different time points during and after routine liver surgery and snap frozen in 2-methylbutane cooled by and stored in liquid nitrogen until further examination.

RNA was extracted and microarray examination of all ischemia samples was performed. Genes were selected which showed RNA profile changes due to pre-examination variation. These changes were further validated by RT-qPCR.

It was observed that some RNA profiles reacted differently from patient to patient under ischaemic conditions, as displayed in Figure A.2 in comparison with a stable RNA profile (see Figure A.1) identified in the study. These unpredictable variations could cause major problems, e.g. in biomarker discovery and development. Hence, the pre-examination workflow should be carefully evaluated and documented.

During the design of the examination system it is strongly recommended to investigate, if the specific RNA profile/s intended to be examined is/are not affected by the entire pre-examination process.

1) Research by the EU FP7 SPIDIA project funded by the European Union Seventh Framework Programme [FP7/2007-2013] under grant agreement no 222916. For further information see www.spidia.eu.