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Molecular in vitro diagnostic examinations — Specifications for pre-examination processes for formalin-fixed and paraffin-embedded (FFPE) tissue —

Part 1: Isolated RNA

Analyses de diagnostic moléculaire in vitro — Spécifications relatives aux processus préanalytiques pour les tissus fixés au formol et inclus en paraffine (FFPE) —

Partie 1: ARN extrait

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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).

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).

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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.

The committee responsible for this document is ISO/TC 212, *Clinical laboratory testing and in vitro diagnostic test systems*.

A list of all parts in the ISO 20166 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.

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, 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 formalin-fixed and paraffin-embedded (FFPE) tissue with regard to RNA examination in what is referred to as the pre-examination phase.

The formalin-fixation and the paraffin-embedding processes lead to modifications of the RNA molecules, which can impact the validity and reliability of the examination test results.

RNA profiles in tissues can change drastically before, during and after collection and change differently in different donors'/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 formalin-fixed and paraffin-embedded (FFPE) tissue —

Part 1: Isolated RNA

1 Scope

This document gives guidelines on the handling, documentation, storage and processing of formalin-fixed and paraffin-embedded (FFPE) tissue specimens intended for RNA examination during the pre-examination phase before a molecular assay is performed.

This document is applicable to molecular in vitro diagnostic examinations including laboratory developed tests performed by medical laboratories and molecular pathology laboratories. 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.

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*

ISO 15190, *Medical laboratories — Requirements for safety*

ISO/IEC 17020:2012, *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/

3.1
aliquot
portion of a larger amount of homogeneous 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 [28], [29] and [30].

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 — EXAMPLE has been removed.]

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 stabilization or fixation

3.6
cDNA
complementary DNA
single-stranded DNA that is complementary to a given RNA synthesized in the presence of reverse transcriptase to serve as a template for synthesis of DNA copies

[SOURCE: ISO 17822-1:2014, 3.12]

3.7
diagnosis
identification of a health or disease state from its signs and/or symptoms, where the diagnostic process can involve *examinations* (3.10) 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

3.8
DNA
deoxyribonucleic acid
polymer of deoxyribonucleotides occurring in a double-stranded (dsDNA) or single-stranded (ssDNA) form

[SOURCE: ISO 22174:2005, 3.1.2]

3.9
DNase
deoxyribonuclease
enzyme that catalyzes the degradation of *DNA* (3.8) into smaller components

3.10
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.]

3.11
formalin
saturated aqueous formaldehyde solution which at 100 % contains 37 % formaldehyde by mass (corresponding to 40 % by volume)

3.12
formalin fixation
treatment of a sample with *standard buffered formalin solution* (3.25) for stabilization

3.13
grossing
gross examination
inspection of pathology specimens with the bare eye to obtain diagnostic information, while being processed for further microscopic examination

3.14
interfering substances
endogenous substances of a *specimen* (3.17)/*sample* (3.23) or exogenous substances (e.g. stabilization solution) that can alter an examination result

3.15
paraffin embedding
process in which a tissue sample is placed in paraffin to generate a hard surrounding matrix so that thin microscopic sections can be cut

3.16
pre-examination process
pre-analytical phase
pre-analytical workflow
process that starts, in chronological order, from the clinician's request and includes 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 ends 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.]

3.17
primary sample
specimen
discrete portion of a body fluid, breath, hair or tissue taken for *examination* (3.10), 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.]

3.18
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.]

3.19
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 or interference

3.20
RNA
ribonucleic acid
polymer of ribonucleotides occurring in a double-stranded or single-stranded form

[SOURCE: ISO 22174:2005, 3.1.3]

3.21
RNase
ribonuclease
enzyme that catalyzes the degradation of RNA into smaller components

3.22
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.

3.23
sample
one or more parts taken from a *primary sample* (3.17)

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

3.24
stability
ability of a sample 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 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.]

3.25
standard buffered formalin solution
neutral buffered formalin
NBF
10 % *formalin* (3.11) solution in water with a mass fraction of 3,7 % (corresponding to a volume fraction of 4 %) formaldehyde buffered to pH 6,8 to pH 7,2

Note 1 to entry: Standard buffered formalin solutions often contain small amounts of methanol to inhibit oxidation and polymerization of formaldehyde.

3.26
storage
prolonged interruption of the pre-analytical workflow of a sample or analyte, or of their derivatives, such as 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.

3.27
tissue processor
automated instrument where tissue fixation, dehydration, clearing and paraffin infiltration occurs

3.28
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: "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.]

3.29
warm ischemia
condition before the tissue is removed from the body, but deprived of its normal blood supply

3.30
workflow
series of activities necessary to complete a task

3.31
homogeneous
uniform in structure and composition

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 analyzed is/are not compromized 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 fixed in standard buffered formalin solution, the RNA profile(s) can change significantly depending on the warm and cold ischemia duration and the temperature before formalin fixation (e.g. gene induction, gene down regulation, RNA degradation). In addition, those changes can vary in different donors'/patients' tissues.

Generally, the longer the durations of warm and cold ischemia and the higher the ambient temperature before fixation of 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 in postoperative cold ischemia[7][8]. RNA profiles can also vary, depending on the origin and type of tissue, the underlying disease, the surgical procedure, 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 (e.g. transport to the laboratory before formalin fixation), its duration shall be documented and the temperatures of the specimen container's surroundings shall be documented. Where the specimen is transported to another facility for formalin fixation, the transport duration shall be documented and the ambient conditions should also be documented.

In addition, the formalin fixation itself as well as the subsequent FFPE tissue storage duration and storage temperature cause modifications of biomolecules and leads to suboptimal analytical test performance of RNA extracted from FFPE tissues (see A.2.1 and A.2.2). This should be considered in the quality control and application of molecular analytical tests, especially in the context of gene expression studies [9][10][11] [12]. These effects can limit the size of amplifiable target cDNA and/or influence the cDNA target sequence of primers used for amplification. Analytical test optimization for FFPE tissues or the use of non-crosslinking alternatives to standard buffered formalin solution are options to minimize this issue for molecular examinations[13][14].

Safety instructions on transport and handling shall be considered and followed in accordance with 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 manufacturers' 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);

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;

d) the documentation steps described under 6.2, if the formalin fixation starts outside the laboratory, and also the documentation steps described under 6.3, if the evaluation of the pathology of the specimen and selection of the sample(s) is also done outside the laboratory.

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

5.1.4 Specimen processing

The following steps shall be performed:

a) the documentation of any addition or modification to the specimen after removal from the body (e.g. labelling for the orientation of the specimen [e.g. ink-marking, stitches, incision(s)]);

b) the selection and use of containers and packages (e.g. cooling box, box for storing and transportation, vacuum packaging) according to applicable transport regulations;

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

NOTE 1 Accidentally freezing the tissue (e.g. by using cool packs in a wrong manner) can lead to RNA degradation when the tissue thaws thereafter. It can also impact the morphological characterization.

NOTE 2 This step can be omitted, if the specimen is transferred directly into standard buffered formalin solution (see 6.2 and notice the importance of volume of fixative and tissue sectioning to allow adequate penetration of fixative).

d) the labelling of the container [e.g. registration-number, barcode (1D or 2D), specimen type, quantity, and organ tissue of origin] and additional documentation [information as specified in 5.1.2, 5.1.3, 5.1.4 a) to c)].

Several specimens from the same patient/donor sharing similar features (macroscopic appearance, tissue type, disease status and 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 cannot be measured, the temperature range should be estimated by classification as ambient temperature, room temperature, or at 2 °C to 8 °C.

5.2 Transport requirements

The laboratory in collaboration with the clinical or surgery department shall establish a protocol for the transport procedure of the specimen.

Temperature monitoring should be applied in a suitable manner.

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

NOTE There is evidence that stabilization of RNA profiles in tissues on wet ice can be further improved by using plastic bags under vacuum[15].

If the specimen is already placed into standard buffered formalin solution outside the laboratory, the temperature during transport should not exceed room temperature.

The conformity 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 ID or 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 Formalin fixation of the specimen or sample(s)

This procedure is applicable to the specimen, and, in case that one or more parts are taken from a specimen, to the resulting sample(s).

The fixative used shall be standard buffered formalin solution.

NOTE In some countries, standard buffered formalin solution is referred to as neutral buffered formalin (NBF).

The pH-value of the standard buffered formalin solution should be checked at least once per week and before use or with every new batch as formalin is not stable (e.g. formaldehyde has a tendency to be oxidized to formic acid)[16].

The following steps shall be performed:

a) the consultation of the manufacturer's Safety Data Sheet (SDS) before handling standard buffered formalin solution;

NOTE Formaldehyde is a carcinogenic and hazardous compound that penetrates the tissue and chemically modifies biomolecules. However, there are potentially different local classifications.

b) the documentation of the time point of placing the tissue specimen or sample into standard buffered formalin solution;

NOTE The total formalin fixation duration can have an impact on further examinations, e.g. immunohistochemical techniques, nucleic acid based molecular examinations[12]; see also A.2.1 and A.2.2. The optimal formalin fixation duration can vary depending on tissue type and size. For larger surgical specimens, e.g. a resected stomach, inhomogeneous fixation can occur before the grossing process due to slow penetration of formaldehyde from the surface of the tissue to the interior.

EXAMPLE For tissue pieces with a thickness of 5 mm, fixation durations between 12 h and 24 h are in most cases reasonable for an appropriate penetration and fixation. See also 6.8.2.

c) the selection of container(s):

1) the capacity of the container(s) should be such that the specimen can be completely submerged into the standard buffered formalin solution. The minimum standard buffered formalin solution to tissue ratio depends on the tissue concerned, but should be at least 10:1 (volume to volume). To ensure complete formalin fixation of larger specimens, a special tissue handling such as incision(s) of solid organs or opening of hollow organs should be performed.

Larger specimens may need to be bisected and appropriate portions selected to ensure adequate fixative penetration. In this case, the standard buffered formalin solution shall be changed periodically.

2) when using containers pre-filled with standard buffered formalin solution, provider's product instructions shall be followed;

3) the container shall be securely closable;

d) the labelling of the container (e.g. by using self-adhesive labels, handwriting, Radio Frequency Identification Devices (RFID), pre-labelled containers, bar codes) shall ensure appropriate traceability of specimens or samples. Therefore, the container labelling shall provide the minimum information of:

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

2) the basic specimen information, e.g. the tissue type, tissue condition, and related additional information 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 6.2 d) 1);

3) the unique numbering of each container, which can be included in 6.2 d) 1);

e) the documentation of types, quantity and description of specimen or samples.

It should be considered that under some disease conditions, such as tumours, molecular features may not be present homogeneously in the tissue specimen or sample. Therefore, it is important that the part of the actual tissue specimen or sample used for molecular examination is evaluated by a medically qualified (e.g. board certified) pathologist (see 6.3). In this context it should be documented which features of a disease are actually reflected in the tissue specimen or sample used for molecular examination (e.g. different molecular mechanisms can be activated in the centre and at the invasion front of the tumour, also tumours can be composed of areas showing different differentiation grades).

6.3 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 histopathological and molecular 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(s) for RNA examination does not compromise the histopathological examination. 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)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 5.1.3).

In the context of the macroscopic evaluation of the surgical specimen before and/or after formalin fixation, 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 shall be checked. The surgical specimen and all findings shall be described appropriately according to the guidelines of the respective medical societies, e.g. societies of pathology, and in correlation with the clinical information and questions, e.g. patient record or clinician's request. 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) Where the tissue specimen was removed from the body without the requirement of a histopathological diagnosis, the documentation of this specimen as well as the evaluation, selection and documentation of the samples may be done by other qualified persons than pathologists.

The documentation can include photographs. The size of the samples shall be appropriate for the tissue cassette (maximum of approximately 3 cm × 2 cm × 0,5 cm). If the specimen is not yet fixed appropriately, post-fixation can be performed within the tissue cassette. Each tissue cassette shall be labelled with a unique identifier (e.g. barcode, number, tissue abbreviation). If a single tissue cassette contains several samples from the same specimen, and the samples represent different features (e.g. tissue type, disease status, location), this shall be documented.

When the sample taken from the specimen is transferred into the tissue cassette, this time point shall be documented.

Without delay, i.e. preferably within 30 min, the sample shall be placed into either standard buffered formalin solution or, if already fixed, it should be placed into an alcohol-containing solution (e.g. 70 % ethanol) on the tissue processor.

The total duration of formalin fixation and the temperature during the fixation process shall be documented.

6.4 Post-fixation of frozen samples

Frozen specimens or samples (e.g. after frozen section diagnosis) can be post-fixed in standard buffered formalin solution for further paraffin embedding.

The total formalin fixation duration shall be documented.

If a formalin-fixed and paraffin-embedded specimen or sample was generated from a frozen specimen or sample, this shall be documented.

6.5 Decalcification

Decalcification adjusts the hard composition of bones to the softness of paraffin. Samples should be decalcified, e.g. with EDTA (ethylenediaminetetraacetic acid). The decalcification procedure shall be documented.

6.6 Processing and paraffin embedding

After the specimen or sample is fixed in standard buffered formalin solution, the time point when it is subsequently placed into an alcohol-containing solution of the tissue processor shall be documented. Further processing shall be performed in a tissue processor according to the manufacturer's instructions.

NOTE 1 During processing, the tissue is dehydrated and water is replaced with paraffin wax. Residual water can affect the quality and stability of tissues, including RNA, during storage[17].

The replacement of all reagents shall be done on a regular basis according to the manufacturer's instructions.

The duration and temperature of paraffin infiltration can impact the biomolecule integrity in fixed tissue. Paraffin with standardized composition and with low melting temperature for tissue infiltration should be used. The duration and temperature of each embedding step shall be performed according to the manufacturers' instructions or laboratories' validated protocols. The applied protocol shall be documented.

NOTE 2 Typical low melting point temperatures for paraffin are in the range from 50 °C to 56 °C.

6.7 Storage requirements

The storage duration and temperature, typically ambient temperature in routine archives, influence the RNA stability in FFPE tissue[18] (see also A.2.4).

RNA degradation occurring during storage can have an impact on the validity and reliability of examination test results[19].

The FFPE tissue should be stored dry at room temperature or preferably at lower temperature.

NOTE 1 Lower storage temperatures (e.g. 2 °C to 8 °C, −20 °C) slow down the RNA degradation process over time[9][18]. See also A.2.4.

NOTE 2 If FFPE tissue is not stored dry, the RNA degradation can increase, and fungal and bacterial growth can be stimulated.

For RNA extractions, FFPE sections should be freshly prepared. If storage of these sections cannot be avoided, they should be stored for as short a duration as possible, dry, refrigerated (at 2 °C to 8 °C) or at lower temperatures (e.g. ≤–20 °C).

Stained and stored sections should not be used, since staining procedures performed on sections can adversely affect RNA quality.

A system for long-term storage of FFPE tissues should be in place. The storage position, storage temperature and the retrieval of any specimen or sample from the storage system, its use, and its return to the storage system shall be documented.

6.8 Isolation of RNA

6.8.1 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[23]). 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.

6.8.2 General information for RNA isolation procedures

Formalin fixation introduces covalent modifications to the RNA by addition of mono-methylol groups[10]. Fixation leads to crosslinking between RNA and proteins. Formaldehyde introduces chemical modifications interfering with enzymatic downstream examination such as RT-PCR.

Requirements and recommendations.

a) The optimal fixation duration depends on the tissue type and thickness. Prolonged tissue fixation results in enhanced RNA modifications and should be avoided. For a tissue thickness of up to 5 mm, the fixation duration should be 12 h to 24 h in standard buffered formalin solution[20].

b) Starting material for RNA purification should be freshly cut sections, with a thickness of up to 10 µm, obtained from FFPE tissue blocks, manually[21] dissected samples, laser microdissected[22] samples or tissue cores [e.g. tissue microarray (TMA)]. Histotechnologists shall wear gloves. The relevant parts of the microtome, including the reusable blade, shall be cleaned after the cutting of each paraffin block. The use of new disposable blades on the microtome should be considered to avoid cross-contaminations.

c) Parallel hematoxylin/eosin (H&E) stained sections should be used to identify, select and control dissection of unstained specimens for subsequent RNA purification. Where RNA purification is intended to be performed with stained material, staining solution should be freshly prepared, using RNase-free reagents to minimize the impact of the staining on RNA quality.

NOTE Staining of tissue can impair RNA quality and performance in later (downstream) examinations.

d) The DNase (see 6.8.4.2), other reagents and consumables coming in touch with the RNA shall be RNase-free.

e) For all RNA isolation procedures from FFPE tissues, measures (e.g. proteinase K digestion and heating) should be included to reverse the formaldehyde modifications such as nucleic acids and protein-RNA crosslinks, without further RNA degradation.

f) 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.

g) To avoid 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.

If RNA is extracted from archived tissue blocks, the blocks should be trimmed by disposing the first sections before taking the sections for RNA isolation, as the outer sections can contain degraded RNA. It may be necessary to further trim blocks in order to enrich for tissue components relevant for examination.

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.

6.8.3 Using commercial kits

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

6.8.4 Using the laboratories' own protocols

If a commercial kit is not used in accordance with 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 independent from a commercial kit, the validation and verification 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.

RNA isolation procedures for FFPE tissue sections should contain the following steps.

a) Removal of paraffin from freshly cut FFPE tissue sections.

NOTE 1 This can be done e.g. by treating with xylene or alternatives, subsequent centrifugation, followed by washing of the resulting pellet with ethanol.

NOTE 2 Commercially available deparaffinization solutions can be used, obviating the need to pellet FFPE tissues.

b) Resuspension of the sections in a lysis buffer, followed by digestion with e.g. proteinase K to remove cross-linked proteins and release the RNA from the sections. Where a heating step is included into the protocol, the lysis buffer should not contain high concentrations of chaotropic salts. The combination of high concentration of chaotropic salt and elevated temperatures can lead to RNA degradation.

NOTE The typical incubation duration can range from 10 min to 18 h (depending on the tissue type and size) at a temperature varying from 37 °C to 60 °C.

It is advised to optimize lysis buffer for the proteinase digestion step[10].

c) Extraction of RNA from the lysate can be performed by mono- or biphasic-extraction, such as phenol/chloroform-based procedures, or by solid phase absorption, such as silica bead procedures[10].

d) A DNase treatment step or other measures to minimize DNA content in the isolated RNA should be incorporated into the RNA isolation procedure. The DNase, other reagents and consumables which come in contact with the sample shall be RNase-free.

For the isolation of RNA from a very low number of cells like laser microdissected single cells, different dedicated RNA isolation procedures might be needed. For such sensitive procedures, it is recommended to follow the latest literature and verify and validate the isolation procedure before use.

6.9 Quantity and quality assessment of isolated RNA

The RNA quantity and quality should be checked according to the diagnostic kit manufacturer's instructions, or according to validated and verified procedures by generally accepted physical, chemical and biochemical procedures[10][11]. These may include one or more of the following techniques, 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 and amplifiability (by e.g. electrophoresis, chromatography, molecular methods such as the 3′/5′assay or differential length amplicon ratio[10] or microfluidic methods to determine quality coefficients [e.g. RNA Integrity Number (RIN), RNA Quality Indicator (RQI)])[12][25][26];

d) test for presence of interfering substances (using exogenous controls [spiked in RNA and DNA controls] or inspecting qPCR response curves for anomalies[26][27] or using an endogenous RNA for an RT-PCR inhibition test by introducing increasing eluate volumes into the examination).

For qualitative examinations, such as presence/absence of a RNA profile 6.9 a) and b) can be sufficient; for quantitative examinations, such as gene expression examination 6.9 a) to d) can be required.

NOTE 1 Formalin fixation has a negative impact on RNA integrity and reliability of RNA quality measurements. Chemical modifications caused by formaldehyde cannot be reliably detected in standard quality control assays such as electrophoretic RNA fragment length measurement, but they interfere with the enzymatic examination.

NOTE 2 Chemical modifications caused by formaldehyde are not reflected by the RIN value calculation. Therefore, measurement of the integrity alone is not sufficient to assess the usability of RNA from FFPE for quantitative RT-PCR.

6.10 Storage of isolated RNA

6.10.1 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.

6.10.2 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.

6.10.3 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 laboratories' own validated RNA isolation procedures are used, the isolated RNA should be assayed immediately. Where the isolated RNA cannot 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.

Depending on the RNA isolation procedure and 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; see also A.2.4. Other validated methods for archiving can also be used[23].

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

Annex A
(informative)

Quality control of RNA extracted from formalin-fixed and paraffin-embedded tissue samples: implications for RT-qPCR based examinations[1)]

A.1 Summary

RNA from different formalin-fixed and paraffin-embedded (FFPE) human liver samples was used to assess the impact of formalin fixation, fixation time and storage of tissue blocks on downstream reactions such as complementary DNA (cDNA) synthesis and reverse transcription quantitative polymerase chain reaction (RT-qPCR) and its implications for quality control in comparison with snap frozen samples which served as reference. The data revealed that formalin fixation led to less efficient cDNA synthesis and RT-qPCR reactions and introduced major gene transcript to gene transcript variations. These differences could not be reliably detected by quality control using routine electrophoresis or spectrophotometry based methods, but could be detected using an RT-qPCR assay based on different amplicon length and a cDNA generation efficiency assay[2)]. Furthermore, RNA degradation depending on storage conditions and fixation time was observed in human samples and by using an animal model.

A.2 Results

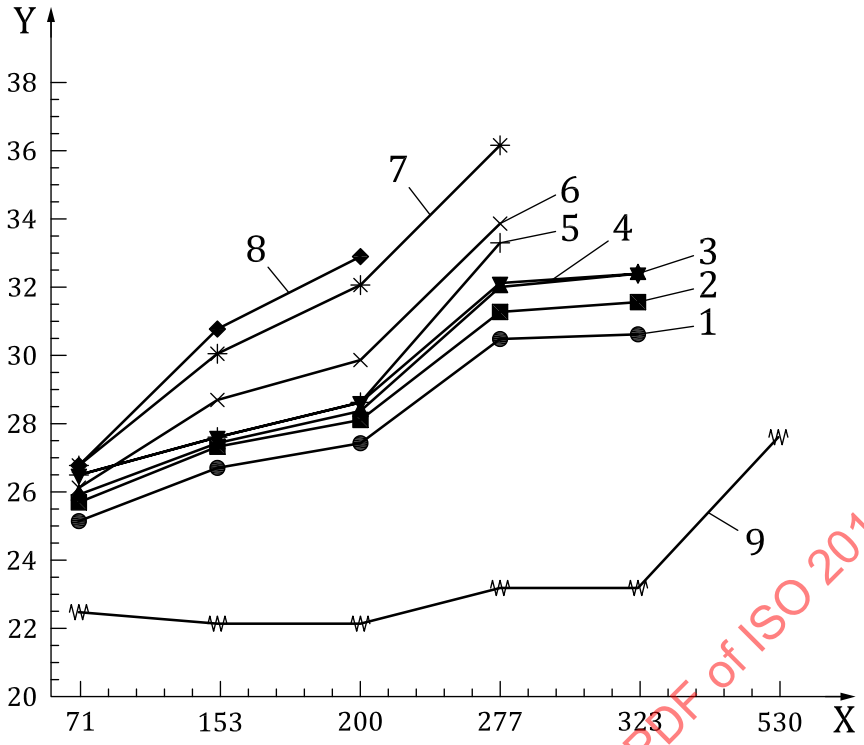
A.2.1 Time dependency of RNA integrity

Formalin fixation impairs RNA integrity in a time-dependent manner as shown in Figure A.1 and Figure A.2

Multiple samples of a human liver sample were fixed for different time periods ranging from 4 h to 120 h in standard buffered formalin solution before paraffin embedding. Similar RNA Integrity Number (RIN) values were obtained for all fixation time points ranging from 2,1 to 2,7. However, the RT-qPCR amplification of fragments of different length of the housekeeping gene GAPDH revealed a rise in cycle threshold (c_t) values for different amplicons correlating with the fixation time. Prolonged fixation induced a steeper slope of the generated curves indicating increased fragmentation and made the amplification of longer fragments impossible.

1) Research by the EU FP/ SPIDIA project funded by the European Union Seventh Framework Programme [FP7/2007-2013] under grant agreement no 222916.

2) Libus J., Storchova H. Quantification of cDNA generated by reverse transcription of total RNA provides a simple alternative tool for quantitative RT-PCR normalization. Biotechniques. 2006, Aug 41 (2), pp. 156, 158, 160 passim



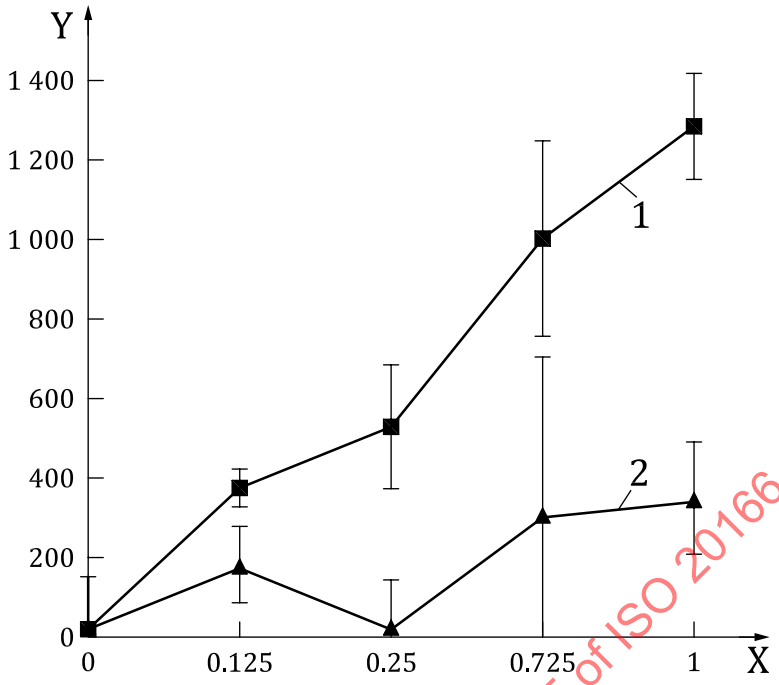
Key

X	amplicon length in bp	5	48 h
Y	c_t value (threshold = 0,2)	6	72 h
1	4 h	7	96 h
2	8 h	8	120 h
3	12 h	9	snap frozen tissue sample
4	24 h		

Figure A.1 — RT-qPCR amplification of GAPDH fragments of different length for human liver samples fixed in standard buffered formalin solution for different time periods before paraffin embedding

A.2.2 Impact of formalin fixation on cDNA synthesis efficiency

The cDNA generation of RNA extracted from snap frozen human liver tissue (see Figure A.2) was in direct correlation to the amount of template RNA. Such correlation is wanted. RNA extracted from FFPE tissue (see Figure A.2) produced only small amounts of cDNA even when more template RNA was provided. The error bars in Figure A.2 depict the standard deviation of the median result in three individual cDNA preparations.

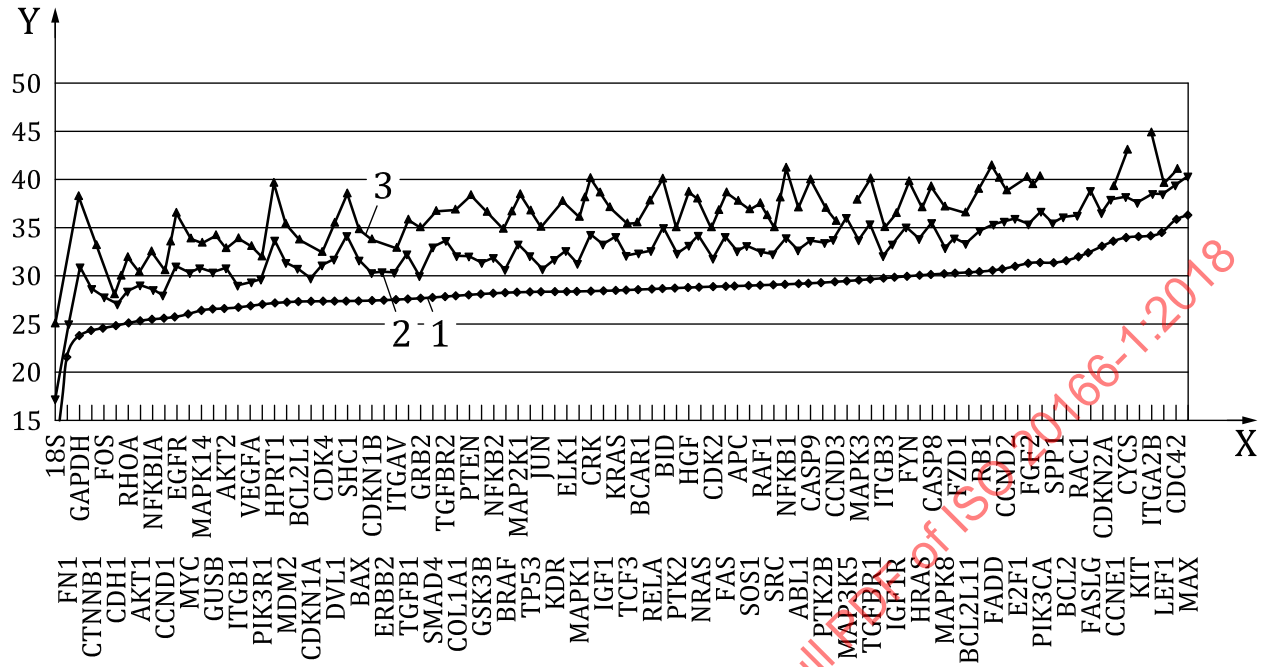


Key

X template RNA in µg
Y relative amount of cDNA
1 snap frozen liver tissue sample
2 FFPE liver tissue sample

Figure A.2 — Generation of extracted cDNA from snap frozen and FFPE human liver tissue samples in correlation to the amount of template RNA

A.2.3 Fixation and storage introduces major gene-to-gene variations in RT-qPCR



Key

X different tested genes

Y *ct* values

1 snap frozen liver tissue sample

2 FFPE liver tissue sample after 6 months

3 FFPE liver tissue sample after 1 year

Figure A.3 — c_t values for 92 genes from snap frozen and FFPE human liver tissue

Samples of a human liver specimen were snap frozen or fixed in standard buffered formalin solution and paraffin embedded. The RNA was extracted from tissue samples at different time points (after 6 months and 1 year). Comparison of RT-qPCR data for 92 genes from snap frozen and FFPE human liver tissue samples revealed an average difference of the c_t values ranging from 4 cycles (6 months) to 8 cycles (1 year) increasing with storage time at room temperature (see Figure A.3). Furthermore, major transcript to gene transcript variations were observed in RNA from all FFPE samples as compared to the snap frozen reference sample. This different and gene-specific behaviour of RNA extracted from FFPE samples could severely impact on the results and interpretation of gene expression studies.