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

Part 3: Isolated DNA

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

Partie 3: ADN extrait

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Contents

Page

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).

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 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 molecular pattern generated during the pre-examination process. Studies have been undertaken to determine the influencing factors for the DNA examination from formalin-fixed and paraffin-embedded (FFPE) tissue. These studies demonstrated that a standardization of the entire process from specimen collection to the DNA examination is needed. This document draws upon such work to codify and standardize the steps for FFPE tissue with regard to DNA examination in what is referred to as the pre-examination phase.

DNA integrity in tissues can change before, during and after formalin fixation, processing and storage. Chemical modifications introduced into DNA during tissue fixation might lead to fragmentation and sequence alterations, changes in the methylation status or even structural changes which can lead to, for instance, spurious copy number changes in array-CGH profiles. These modifications of the DNA molecules can impact the validity and reliability of the examination test results. Therefore, it is essential to take special measures to minimize the described DNA changes and modifications for subsequent examination.

In this document, the following verbal forms are used:

— "shall" indicates a requirement;

— "should" indicates a recommendation;

— "may" indicates a permission;

— "can" indicates a possibility or a capability.

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

Part 3: Isolated DNA

1 Scope

This document gives guidelines on the handling, documentation, storage and processing of formalin-fixed and paraffin-embedded (FFPE) tissue specimens intended for DNA 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*

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 [25], [26], and [27].

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, specificity 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
diagnosis
identification of a health or disease state from its signs and/or symptoms, where the diagnostic process can involve *examinations* (3.8) 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
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.]

3.9
formalin
saturated aqueous formaldehyde solution which at 100 % contains 37 % formaldehyde by mass (corresponding to 40 % by volume)

3.10
formalin fixation
treatment of a *sample* (3.18) with *standard buffered formalin solution* (3.20) for stabilization

3.11
grossing
gross examination
inspection of pathology specimens with the bare eye to obtain diagnostic information, while being processed for further microscopic examination

3.12
interfering substances
endogenous substances of a *specimen* (3.15)/*sample* (3.18) or exogenous substances (e.g. stabilization solution) that can alter an examination result

3.13
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

3.14
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)* (3.15), transportation to and within the medical or pathology laboratory, isolation of *analytes* (3.3), 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.]

3.15
primary sample
specimen
discrete portion of a body fluid, breath, hair or tissue taken for *examination* (3.8), 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.]

3.16
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.]

3.17
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.

3.18
sample
one or more parts taken from a *primary sample* (3.15)

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

3.19
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 DNA.

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

3.20
standard buffered formalin solution
neutral buffered formalin
NBF
10 % *formalin* (3.9) 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 polymerisation of formaldehyde.

3.21
storage
prolonged interruption of the *pre-analytical workflow* (3.14) of a *sample* (3.18) or *analyte* (3.3) respectively, 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.

3.22
tissue processor
automated instrument where tissue fixation, dehydration, clearing and paraffin infiltration occurs

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

3.25
workflow
series of activities necessary to complete a task

3.26
homogeneous
uniform in structure and composition

4 General considerations

For general statements on medical laboratory quality management systems and in particular on specimen collection 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 for 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.

In contrast to RNA or proteins, DNA in tissue is relatively stable during warm and cold ischemia. Changes of DNA sequence or copy numbers [e.g. comparative genomic hybridization (CGH) profiles] due

to longer warm and cold ischemia durations are unknown[7]. However, DNA methylation patterns may change in response to ischemia[6]. The duration until the specimen is placed into standard buffered formalin solution should be kept as short as possible in order to avoid enzymatic degradation of DNA. The duration before fixation shall be documented and the temperature before fixation should be documented[5].

During the fixation, processing and storage, the DNA integrity can change depending on the kind of fixative, fixation time and temperature, storage or archiving of the fixed paraffin-embedded tissue as well as the method used for DNA isolation and purification. When using a fixative based on formaldehyde, temperature and fixation duration have a significant impact on DNA integrity. The longer the fixation duration and the higher the temperature, the more chemical modifications and crosslinks are introduced, which can lead to degradation or sequence alterations[9][10][11][12][13][14]. These effects can limit the size of amplifiable target DNA and/or influence the target sequence of primers used for amplification.

Safety instructions on specimen 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 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 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)];

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 DNA 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 collection transport 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 transport 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 DNA degradation.

The temperatures of the transport 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 changes to the DNA.

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

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 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.

The correct identity of the specimen shall be checked. This should include the clinical information (see 5.1.2 and 5.1.3) of the specimen, 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 the event 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)[13].

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 potential different local classifications.

b) the documentation of the time point of placing the tissue 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[16]; see also A.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 collection containers 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)[7]. 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, collection 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 or sample 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 specimens 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. Tumours can also 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 DNA examination.

a) The selection of appropriate parts of the specimen for molecular and histopathological examinations as well as for optional further research purposes shall be done by or under supervision of a medically qualified (e.g. board certified) pathologist to ensure that the collection of the sample(s) for DNA 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. 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 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 should 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) When 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.

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 of 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 60 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 an FFPE 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 by paraffin wax. Residual water can affect the quality and stability of tissues, including DNA, during storage[7].

The replacement of all reagents shall be done on a regular basis according to the manufacturers' 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 DNA stability in FFPE tissue[29] (see A.2).

The FFPE tissue block 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 DNA degradation process over time (see also Annex A).

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

NOTE 3 There is evidence that the FFPE blocks can be analysed after 5 years or longer, if they are processed and stored appropriately.

For DNA 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.

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 DNA

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

6.8.2 General information for DNA isolation procedures

Formalin fixation introduces covalent modification to DNA by addition of mono-methylol groups. In a second step electrophilic addition of N-methylol on an amino base leads to the formation of methylene bridges between two amino groups.

Formaldehyde introduces chemical modifications interfering with enzymatic reactions in subsequent molecular examinations e.g. DNA polymerase.

Requirements and recommendations.

a) The optimal fixation duration depends on the tissue type and size. Prolonged tissue fixation results in irreversible DNA 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.

b) Starting material for DNA purification should be freshly cut sections, with a thickness of up to 10 µm, obtained from FFPE tissue blocks, manually[17] dissected samples, laser microdissected[18] samples or tissue cores [for 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 DNA purification.

NOTE Staining can impair DNA quality and performance in later (downstream) analytical applications.

d) For all DNA isolation procedures, measures should be included to reverse as many of the formaldehyde modifications as possible without further DNA degradation:

— Digestion with, for example, proteinase K and heating partially reverses formaldehyde-introduced modifications of nucleic acids and protein-DNA crosslinks.

NOTE 1 Alterations of DNA in FFPE tissues include base modifications and deamination which upon PCR or primer extension may lead to erroneous read-out.

NOTE 2 Most frequent is a C:G to T:A transitional change which can be reversed to some extent by treatment of FFPE DNA with uracil-DNA glycosylase[19] after or during DNA purification.

e) To avoid cross contamination with amplified material from the DNA analytical test, the isolation of the DNA should not be performed in the same area as the amplification, unless a closed system is used.

If DNA is extracted from archived tissue blocks, the blocks should be trimmed by disposing of the first sections before taking the sections for DNA isolation, as the outer sections can contain degraded DNA. 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 DNA is a key step in the diagnostic workflow, which shall be especially focused on during the validation of the entire workflow.

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

6.8.3 Using commercial kits

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

6.8.4 Using the laboratories' own protocols

6.8.4.1 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.

6.8.4.2 If the laboratory uses its own protocol independent from a commercial kit, the verification and 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 in combination for diagnostic testing only if the components have been tested together and validated to work satisfactorily.

DNA 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 with solvents such as xylene. Alternatively, high temperature methods can be used, which release tissue from paraffin but avoid the need for solvents.

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 is followed by digestion with, for example, proteinase K to remove cross-linked proteins and to release the DNA from the sections.

It is advised to optimize lysis buffer for the proteinase digestion step and the following heating step.

NOTE The typical digestion period with proteinase K is 1 h to 18 h, at a temperature in a range from 37 °C to 60 °C.

c) Heat incubation for up to 1 h at up to 90 °C to remove the majority of methylol-additions and to reverse cross-links.

NOTE Longer incubation durations or higher incubation temperatures can result in more fragmented DNA.

d) Optional: If RNA-free genomic DNA is required, incubation with RNase A shall be performed.

NOTE 1 For laboratory developed methods, it can be required to incorporate an RNase digestion step, if a too high amount of RNA, disturbing the examination test, is co-purified with the DNA.

NOTE 2 Co-purified RNA, even after digestion, can lead to over quantification of DNA yield by spectrophotometric methods. This over quantification and the actual presence of RNA can also interfere with the examination test.

e) Extraction of the DNA from the lysate, e.g. by phenol/chloroform based methods or by using commercially available kits for DNA purification from FFPE tissue.

6.9 Quantity and quality assessment of isolated DNA

The DNA quantity and quality should be checked according to the diagnostic kit manufacturer's instructions, or according to verified and validated procedures by generally accepted physical, chemical and biochemical procedures. These may include one or more of the following techniques, depending on the specific examination test:

a) quantification by absorbance measurements (A_{260}), spectrofluorometry or a genome specific qPCR assay[20];

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

c) test for DNA integrity and amplifiability (by e.g. electrophoresis, chromatography, or molecular methods such as the differential length amplicon ratio)[21][22];

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

For qualitative examinations, such as presence/absence, sequencing, copy number variation 6.9 a) and b) are often applied; for quantitative examinations 6.9 a) to d) can be required.

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

6.10 Storage of isolated DNA

The specific instructions supplied by the DNA isolation kit provider for storing isolated DNA should be followed. Where the examination provider's instructions are more stringent than the specific instructions supplied by the DNA isolation kit provider, e.g. lower temperature, the examination provider's instructions shall be followed.

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

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

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

Storage for long-term purposes should be at –20 °C or below. Other validated methods for archiving can also be used[23].

Appropriate storage vessels, such as cryogenic vials, should be used.

For long-term storage, aliquots of the isolated DNA should be generated to avoid repeated freezing and thawing.

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

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