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

Part 4: In situ detection techniques

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

Partie 4: Techniques de détection in situ

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Foreword

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

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

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

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

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

This document was prepared by Technical Committee ISO/TC 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 by new technologies analyzing tissue morphology and biomolecules, such as (e.g. proteins, DNA, RNA and/or metabolites (e.g. glucose) in human tissues and body fluids.

In pathology, the majority of diagnoses are based on in situ staining of formalin-fixed and paraffin-embedded (FFPE) tissue sections. In the context of personalized medicine, classical histological staining (e.g. hematoxylin and eosin) for morphological evaluation is increasingly complemented by additional in situ detection techniques, such as immunohistochemistry or in situ hybridization, as well as molecular analysis of isolated biomolecules. For example, many regulatory bodies approved companion diagnostics in oncology are based on in situ detection techniques applied on FFPE tissue sections. Developments in personalized medicine and new technologies, such as multi-label immunostaining and computer-based analysis of digital images (e.g. generated by using a slide scanner) pose new requirements on standardization of pre-analytical procedures to obtain reproducible qualitative and quantitative results.

Profiles and/or integrity of biomolecules and their in situ localization, amount and accessibility for in situ detection in tissues can change drastically during the pre-examination process comprising specimen collection, tissue processing, embedding, transport, storage, sectioning and pretreatment for in situ detection. This makes the outcome from in situ detection in diagnostics or research unreliable or even impossible because the subsequent examination will not represent the in vivo state of molecules, but instead, an artificial profile or morphology generated during the pre-examination process.

Therefore, a standardization of the entire pre-examination process of FFPE tissue specimens intended for in situ examinations of morphology and biomolecules on FFPE tissue sections by using different in situ detection techniques, is needed.

There is multiple scientific evidence that several factors of the pre-examination phase influence the outcome (e.g. quality or quantity in terms of specificity or sensitivity) of in situ detection and, thus, can have major impact on the diagnostic results.

This document draws upon such work to organize and standardize the steps for formalin-fixed and paraffin-embedded (FFPE) tissue with regard to various in situ detection techniques in what is referred to as the pre-examination phase. This document is for the pre-examination phase of in situ detection techniques and is applicable to the whole spectrum of in situ detection techniques.

These include but are not limited to:

— Classical histological staining, e.g. Hematoxylin & Eosin staining (H&E);

— Histochemical techniques, e.g. Lipid staining, Periodic Acid Schiff (PAS) reaction, Perls' Prussian Blue reaction, Feulgen's reaction, enzyme histochemistry;

— Immunohistochemical staining (IHC) or immunofluorescence staining using antibodies (polyclonal, monoclonal or recombinant antibodies) or other affinity binders;

— Hybridization-based techniques such as RNA or DNA in situ hybridization (ISH) techniques, e.g. fluorescence in situ hybridization (FISH), chromogenic in situ hybridization (CISH), or silver enhanced in situ hybridization (SISH);

— Molecular analysis of isolated biomolecules that can be mapped to a defined region of an FFPE section (by e.g. in situ sequencing, imaging mass spectrometry).

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 preexamination processes for formalin-fixed and paraffin-embedded (FFPE) tissue —

Part 4: In situ detection techniques

1 Scope

This document specifies requirements and gives recommendations for the collection, handling, documentation, transport, storage and processing during the pre-examination phase of formalin-fixed and paraffin-embedded (FFPE) tissue specimens intended for qualitative and/or (semi-)quantitative in situ examination of the morphology and of biomolecules, such as metabolites, proteins, DNA and/or RNA, on FFPE tissue sections by using different in situ detection techniques.

This document is applicable to in vitro diagnostic examinations using in situ detection techniques. These include laboratory developed tests performed by pathology laboratories (histopathology laboratories) as well as by molecular pathology laboratories and other medical laboratories. It is also intended to be used by laboratory customers, in vitro diagnostics developers and manufacturers, biobanks, as well as institutions and commercial organizations performing biomedical research, and regulatory authorities.

This document is not applicable to the pre-examination phase of RNA, proteins and DNA isolated from FFPE tissue for examination. These are covered in ISO 20166-1, ISO 20166-2 and ISO 20166-3, Molecular in vitro diagnostic examinations — Specifications for pre-examination processes for isolated RNA, proteins and DNA, respectively.

Different dedicated measures are taken for pre-examination processes for fine needle aspirates (FNAs). These are covered in CEN WI 00140128, CEN WI 00140126, and CEN WI 00140129, Molecular in vitro diagnostic examinations — Specifications for pre-examination processes for Fine Needle Aspirates (FNAs) isolated cellular RNA, isolated proteins, and isolated genomic DNA, respectively.

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

2 Normative references

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

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

ISO 15190, *Medical laboratories — Requirements for safety*

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
affinity binder
molecules including affibodies, peptides, *antibody* (3.3) fragments or other small molecules that interact with *biomolecules* (3.6) and structures in a cell, and can be used in *in situ detection* (3.27) techniques

3.2
ambient temperature
unregulated temperature of the surrounding air

[SOURCE: ISO 20166-1:2018, 3.2]

3.3
antibody
protein (3.36) (immunoglobulin) produced and secreted by B lymphocytes in response to a molecule recognized as foreign (*antigen* (3.4)) and which is capable of binding to that specific *antigen* (3.4)

[SOURCE: ISO 16577:2016, 3.10, modified — "Note 1 to entry" has been deleted.]

3.4
antigen
substance that stimulates the production of *antibodies* (3.3) and reacts with them

[SOURCE: ISO 15089:2000, 3.5]

3.5
antigen retrieval
epitope retrieval
procedure(s) to unmask *antigens* (3.4)/*epitopes* (3.14) and restore their binding properties for *antibodies* (3.3) used in *immunohistochemistry* (3.25) by neutralizing the modifications introduced by *formalin fixation* (3.19), *tissue processing* (3.47) and *paraffin embedding* (3.33) of tissue

3.6
biomolecule
organic molecule produced in living organisms that is involved in the maintenance and metabolic processes of organisms

Note 1 to entry: The examples of organic molecule are *protein* (3.37), carbohydrate, lipid, or nucleic acid.

3.7
clearing
process step in *tissue processing* (3.47) in which formalin-fixed tissue is transferred from *dehydration* (3.10) reagent to clearing agent (for example, xylene) to prepare the tissue for *impregnation* (3.26)

3.8
cold ischemia
condition after removal of the tissue from the body until its stabilization or fixation

[SOURCE: ISO 20166-1:2018, 3.5]

3.9
decalcification
technique using chemical agents for removal of mineral (inorganic calcium) from bone or other calcified tissue to adjust the hard tissue components to the softness of *paraffin* (3.32) for sectioning

3.10
dehydration
process step in *tissue processing* (3.47) for removal of water from formalin-fixed tissue by immersing the tissue in a series of dehydrating reagent solutions of increasing concentration finishing with water free (100 %) solution

3.11
deviation
departure from an approved instruction, procedure and/or method

[SOURCE: ISO 15378:2017, 3.7.5 modified — "approved (3.7.1) standard operating procedure (SOP) (3.7.10)" replaced by "approved instruction, procedure and/or method".]

3.12
diagnosis
identification of a health or disease state from its signs and/or symptoms, where the diagnostic process can involve *examinations* (3.15) and tests for classification of an individual's condition into separate and distinct categories or subclasses that allow medical decisions about treatment and prognosis to be made

[SOURCE: ISO 20166-1:2018, 3.7]

3.13
DNA
deoxyribonucleic acid
polymer of deoxyribonucleotides occurring in a double-stranded (dsDNA) or single-stranded (ssDNA) form

[SOURCE: ISO 22174:2005, 3.1.2]

3.14
epitope
antibody (3.3) binding site on a *biomolecule* (3.6) that is an *antigen* (3.4)

3.15
examination
analytical test
set of operations with the objective of determining the value or characteristics of a property

Note 1 to entry: Processes that start with the *in situ detection* (3.27) using *antibodies* (3.3), nucleic acid probes or dyes 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.]

3.16
examination manufacturer
analytical test manufacturer
entity that manufactures and/or produces the specific *analytical test* (3.15)

3.17
FFPE tissue
formalin-fixed, paraffin-embedded tissue
tissue *specimens* (3.42)/*samples* (3.41) having undergone fixation in *formalin* (3.18, 3.19), *tissue processing* (3.47), and *paraffin embedding* (3.33) in a tissue cassette

3.18
formalin
saturated aqueous formaldehyde solution which at 100 % contains 37 % formaldehyde by mass (corresponding to 40 % by volume)

[SOURCE: ISO 20166-1:2018, 3.11]

3.19
formalin fixation
treatment of a *sample* (3.41) with *standard buffered formalin solution* (3.45) for stabilization

[SOURCE: ISO 20166-1:2018, 3.12]

3.20
formalin pigment
acid formalin haematin pigment acid
hematin
black to brown amorphous to microcrystalline granules representing an artefact in histologic sections prepared from tissues fixed in *formalin* (3.18, 3.19) having an increased formic acid concentration, which is produced by acid acting upon haemoglobin

3.21
grossing
gross examination
inspection of pathology *specimens* (3.42) with the bare eye to obtain diagnostic information, while being processed for further microscopic *examination* (3.15)

[SOURCE: ISO 20166-1:2018, 3.13]

3.22
histochemical technique(s)
in situ detection (3.27) technique(s) for the visualization and characterization of *biomolecules* (3.6) that involves chemical reactions with specific groups, radicals, or chemical bonds in *biomolecules* (3.6) and provide(s) information on the *biomolecules'* (3.6) in situ localization in *tissue sections* (3.49)

Note 1 to entry: The examples of *biomolecules* (3.6) are carbohydrates, lipids, other metabolites, *proteins* (3.36), amino acids, nucleic acids, pigments, or enzymes etc.

3.23
histological staining
in situ detection (3.27) technique undertaken to prepare *tissue sections* (3.49) by using histological stains (e.g. Haematoxylin-Eosin (HE), Chromotrop-Anilinblue (CAB)) to highlight features of the *tissue section* (3.49) and enhance *tissue section* (3.49) contrast

3.24
homogeneous
uniform in structure and composition

[SOURCE: ISO 20166-1:2018, 3.31]

3.25
immunohistochemistry
IHC
in situ detection (3.27) technique that uses the principle of *antibodies* (3.3) binding specifically to *antigens* (3.4) / *epitopes* (3.14) in biological tissues or cells to visualize *antigens* (3.4) (e.g. *proteins* (3.36)) using brightfield microscopy

3.26
impregnation
impregnation with paraffin
process step in *tissue processing* (3.47) for replacement of clearing agent and infiltration of tissue with molten *paraffin* (3.32)

3.27
in situ detection
technique that allows for precise localization of a specific *biomolecule* (3.6) within a slide-mounted section

EXAMPLE *Immunohistochemistry* (3.25) for *protein* (3.36) detection, *in situ hybridization* (3.30) for nucleic acid detection, *histological staining* (3.23), *histochemical techniques* (3.22), MALDI imaging mass spectrometry, imaging mass cytometry, in situ Raman spectroscopy.

3.28
in situ detection system
detection system
set of reagents used to visualize the binding of molecules to their target in situ within a *tissue section* (3.49)

Note 1 to entry: Examples of molecules are *antibody* (3.3), *affinity binder* (3.1), and nucleic acid.

Note 2 to entry: Most common for *in situ detection* (3.27) are enzyme- and fluorophore-based detection systems.

3.29
in situ examination
analytical in situ test
examination (3.15) based on *in situ detection* (3.27)

3.30
in situ hybridization
ISH
in situ detection (3.27) technique that uses the principle of complementary nucleic acid probes binding specifically to segments of *RNA* (3.37) or *DNA* (3.13) in biological tissues or cells to visualize nucleic acids (e.g. *DNA* (3.13) or *RNA* (3.37)) using brightfield or fluorescence microscope

3.31
nonconformity
nonfulfillment of a requirement

[SOURCE: ISO 9000:2015, 3.6.9 modified — Note 1 removed.]

3.32
paraffin
paraffin wax
product obtained from distillates, consisting essentially of a mixture of saturated hydrocarbons, solid at *room temperatures* (3.40) used for *paraffin embedding* (3.33) of formalin-fixed tissue *specimens* (3.42)/*samples* (3.41)

3.33
paraffin embedding
embedding in paraffin
process following *tissue processing* (3.47) in which a tissue *sample* (3.41), is placed in melted *paraffin* (3.32) to achieve a hard surrounding matrix so that thin microscopic sections can be cut

3.34
post-translational modification
modifications on a *protein* (3.36), catalysed by enzymes, after its translation by ribosomes is complete that can be the addition of a functional group covalently to a *protein* (3.36) such as phosphorylation and neddylation, the proteolytic processing and/or folding processes necessary for a *protein* (3.36) to mature functionally

3.35
pre-examination process
pre-analytical workflow
pre-analytical phase
pre-examination phase
process that starts, in chronological order, from the clinician's request and includes the *examination* (3.15) request, preparation and identification of the patient, collection of the *speciment(s)* (3.42), transportation to and within the medical laboratory, evaluation, *tissue processing* (3.47), *paraffin embedding* (3.33), FFPE block *storage* (3.46) and retrieval, sectioning (including mounting onto glass slides and drying), *storage* (3.46) of unstained slide-mounted *tissue sections* (3.49) (if applicable), and pre-treatment steps for *in situ detection* (3.27) techniques, and ends when the analytical *examination* (3.15) begins

Note 1 to entry: The pre-examination phase for *in situ detection* (3.27) includes preparative processes, e.g. *antigen/epitope retrieval* (3.5) and pre-hybridization procedures, which influence the outcome of the intended *examination* (3.15).

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

3.36
protein
type of biological *biomolecules* (3.6) composed of one or more chains with a defined sequence of amino acids connected through peptide bonds

[SOURCE: ISO 20166-2:2018, 3.14, modified – The terms "biological macromolecules" were replaced with "biological biomolecules"]

3.37
RNA
ribonucleic acid
polymer of ribonucleotides occurring in a double-stranded or single-stranded form

[SOURCE: ISO 20166-1:2018, 3.20]

3.38
RNA profile
amounts of the individual *RNA* (3.37) molecules that are present in a *sample* (3.41) and that can be measured in the absence of any losses, inhibition and interference

[SOURCE: ISO 20166-1:2018, 3.19]

3.39
RNase
ribonuclease
enzyme that catalyzes the degradation of *RNA* (3.37) into smaller components

[SOURCE: ISO 20166-1:2018, 3.21]

3.40
room temperature
for the purpose 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.

[SOURCE: ISO 20166-1:2018, 3.22]

3.41
sample
one or more parts taken from a *specimen* (3.42)

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

3.42
specimen
primary sample
discrete portion of a body fluid, breath, hair or tissue taken for *examination* (3.15), study or analysis of one or more quantities or properties assumed to apply for the whole

[SOURCE: ISO 15189:2012, 3.16, modified — The term and definition is used here without the original notes.]

3.43
specimen container
container into which the tissue *specimen* (3.42) is transferred after its collection for further *pre-analytical workflow* (3.35) steps (e.g. transport and/or fixation)

3.44
stability
ability of a *sample* (3.41) 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 analytes for the purpose of this document are *biomolecules* (3.6) located in situ/in the tissue.

[SOURCE: ISO 20166-1:2018, 3.24, modified — Note 1 to entry has been deleted and new Note 1 to entry has been added.]

3.45
standard buffered formalin solution
neutral buffered formalin
NBF
10 % *formalin* (3.18) 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.

[SOURCE: ISO 20166-1:2018, 3.25]

3.46
storage
maintenance of biological material under appropriate conditions for intended use

3.47
tissue processing
process during the generation of *FFPE tissue* (3.17) consisting of the four consecutive steps tissue fixation, *dehydration* (3.10), *clearing* (3.7), and *impregnation with paraffin* (3.26)

3.48
tissue processor
automated instrument where tissue fixation, *dehydration* (3.10), *clearing* (3.7) and *impregnation (with paraffin)* (3.26) occur

[SOURCE: ISO 20166-1:2018, 3.27]

3.49
tissue section
very thin slice of tissue generated by cutting tissue with a dedicated section cutting device (i.e. microtome)

Note 1 to entry: *FFPE tissue* (3.17) is cut using a microtome to generate (µm-) thin sections that are mounted onto glass slides for performing *in situ detection* (3.27) techniques.

3.50
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 word "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.]

3.51
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.

Note 2 to entry: Confirmation can comprise activities such as

— performing alternative calculations,

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

— undertaking tests and demonstrations, and

— reviewing documents prior to issue.

[SOURCE: ISO 9000:2015, 3.8.4]

3.52
warm ischemia
condition before the tissue is removed from the body, but deprived of its normal blood supply

Note 1 to entry: Disruption of normal blood supply varies significantly case by case. Therefore, warm ischemia duration and its effects depend on individual cases and it also can depend on the anatomy of the arterial blood supply.

[SOURCE: ISO 20166-1:2018, 3.29]

3.53
workflow
structured series of activities necessary to complete a task

[SOURCE: ISO 20166-1:2018, 3.30, modified - The word "structured" was added]

4 General considerations

For general statements on medical laboratory quality management systems and in particular on specimen collection, receipt, and handling (including avoidance of cross contaminations) see ISO 15189, ISO/IEC 17025 or ISO/IEC 17020. ISO/TS 20658 and ISO 20387 (for biobanking) can also apply. The requirements on laboratory equipment, reagents, and consumables according to ISO 15189 shall be followed; ISO/IEC 17025 and ISO/IEC 17020 can also apply.

All steps of the pre-examination, examination and post-examination processes (i.e. the entire workflow) can influence the diagnosis or research study results. Thus, this entire workflow shall be verified and validated. This includes specifically all pre-examination process steps such as the examination request, preparation and identification of the patient, collection of the primary sample(s), transportation to and within the medical laboratory, fixation, processing, embedding, sectioning and storage.

Pre-analytical workflow steps, which cannot always be controlled (e.g. duration and condition of warm ischemia depending on the surgical procedure), shall be documented. A risk assessment of pre-analytical

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.

NOTE 1 Disruption of normal blood supply varies significantly case by case. Therefore, warm ischemia duration and its effects depend on individual cases and it can also depend on the anatomy of the arterial blood supply.

The preservation of tissue morphology and the stability of the specific biomolecules of interest and their post-translational modifications (if important for the analytical test) should be investigated throughout the complete pre-examination process prior to the development and implementation of an analytical test (e.g. by performing a time course experiment or study; see references[2-4]).

Before tissues are fixed in standard buffered formalin solution, biomolecule in situ/subcellular localization, amounts, conformations and binding status can change, e.g. by biomolecule degradation, washing out, crosslinking and altered synthesis following e.g. gene induction, gene down regulation, RNA degradation, changes in post translational modification of proteins and changes of the biochemical pathway and energy status. These effects depend on the duration of warm and cold ischemia, and the temperature before formalin fixation. In addition, the described effects can vary in different patients'/donors' tissues.

Generally, the longer the durations of warm and cold ischemia and the higher the temperature before fixation of the tissue specimen, the higher is the risk that changes in biomolecule profile and in situ/subcellular localization can occur.

NOTE 2 Biomolecule amounts, modifications, and in situ/subcellular location can also vary, depending on the origin and type of tissue, the underlying disease, the surgical procedure, the drug regime, and drugs administered for anaesthesia or treatment of concomitant disease, and on the different environmental conditions after the tissue removal from the body. Prolonged cold ischemia results in changes of protein and phosphoprotein amounts[5,6,48]. Keeping the specimen at low temperature (e.g. 4° C diminishes this effect.[7] RNA profiles 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)[8,9]. Changes of DNA, sequence or copy numbers (e.g. comparative genomic hybridization (CGH) profiles) due to longer warm and cold ischemia durations are unknown[10]. However, DNA methylation patterns can change in response to ischemia[11].

Where relevant, warm ischemia duration shall be documented (e.g. for large tumor resections). Where it is not possible to avoid cold ischemia (e.g. due to transport to the laboratory before formalin fixation), its duration shall be documented, and the temperatures of the specimen container's surroundings shall be documented.

Formalin fixation (including quality of formalin solution, ratio of sample volume to formalin solution volume, duration and temperature of fixation) and other pre-examination processes can impact morphology,[12] cause modifications of biomolecules, and lead to suboptimal analytical test performance affecting results obtained by in situ detection. These pre-examination processes include tissue processing,[13] embedding,[13] storage of FFPE tissue blocks,[13] sectioning (including selection of types of slides, section thickness, drying and storage of unstained slide mounted tissue sections). This should be considered in the quality control and application of in situ detection tests. Optimization of the analytical test for FFPE tissues and/or of the pretreatment of slide mounted FFPE tissue sections (e.g. antigen retrieval, pre-hybridization; see 6.10.2 and 6.10.3) are options to minimize this issue for in situ detection examinations.

Immunohistochemistry can be affected by modification or loss of immunoreactivity in FFPE tissue due to denaturation of molecules,[14] crosslinking of proteins, and thus loss or "masking" of epitopes or steric interference of antibody binding to epitopes[3,15]. Evidence shows that many antigens (epitopes) can substantially, if not completely, be restored by antigen retrieval[16,17]. Therefore, antigen retrieval can be performed in order to restore immunoreactivity in FFPE tissue and/or to "retrieve" epitopes "masked" or modified by formalin fixation, and tissue processing and embedding. Since optimal antigen retrieval procedures depend on the pre-analytical conditions applied, antigen retrieval requirements shall be verified in the context of the whole pre-analytical and analytical workflow.

NOTE 3 Extensive antigen retrieval can cause morphological artefacts.

In situ hybridization can be affected by biomolecule properties such as nucleic acid integrity, nucleic acid–nucleic acid interaction or nucleic acid–protein interaction, which can be reversed by pre-hybridization procedures optimized for the specific pre-analytical conditions. Therefore, pre-hybridization procedure requirements shall be verified in the context of the entire workflow required for the specific examination.

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

Safety instructions for the whole pre-examination process shall be in place and followed. Safety requirements on specimen collection, transport and handling shall be according to relevant ISO standards such as ISO 15189 and ISO 15190.

If a commercial product is not used in accordance with the manufacturer's instructions due to unmet patient needs, the laboratory shall verify and validate for its intended use. The responsibility and risk for its use lies with the user. See Annex A.

5 Activities 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 examination (see also Clause 6) should be considered.

The laboratory in collaboration with the clinical or surgery department and doctor practices shall establish instructions for the required documentation of the patient/specimen donor, collection of the specimen, selection and labelling of the specimen container and transport procedures for the specimen.

See also ISO 15189.

5.1.2 Information about the patient/specimen donor

The documentation shall include the identity of the patient/specimen donor, which can be in the form of the name or a code.

The documentation should include, but is not limited to:

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

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

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

5.1.3 Information about the specimen

The documentation shall include, but is not limited to:

a) for surgical specimens: if required for the examination the start of ischemia within the body (warm ischemia) by documentation of the ischemia-relevant vessel ligation/clamping time point (usually arterial clamping time) to allow for calculation of the warm ischemia duration, which ends with excision of the specimen and removal from the body (see 6.6). For documentation of warm ischemia duration, cooperation with operating theatre staff is required.

NOTE For biopsies warm ischemia is negligible.

b) for autopsy specimens: the estimated time point of death (for defining the post-mortem interval);

c) the time point and date when tissue is removed from the body [to allow for calculation of the cold ischemia duration, which ends with start of fixation (see 6.2)], and the method of removal (e.g. core-needle biopsy, resection, biopsy device used for the collection);

d) 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; The documentation should also include the identity (e.g. name or code) of the responsible person for collecting the specimen.

5.1.4 Specimen processing, intermediate storage and preparation for transport

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), measures to avoid drying of the specimen e.g. moisturising with isotonic buffer to prevent drying of the specimen surface];

b) the selection and use of specimen containers and packages (e.g. closable container, vacuum packaging; cooling box, box for storing and transportation) used for transport according to applicable transport regulations; the specimen container used shall be securely closable. When using specimen containers pre-filled with standard buffered formalin solution, the manufacturer's instructions shall be followed.

c) the selection and use of either a formalin fixation procedure (as described in 6.2), or, where formalin fixation of the tissue occurs in the laboratory, of stabilization procedures (e.g. vacuum-packaging, cooling methods) for transport; the documentation of steps described under 6.2 (including documentation of date and time point of placing the tissue in formalin), if the formalin fixation starts outside the laboratory; the documentation steps described under 6.3, if the evaluation of the pathology of the specimen and the selection of the sample(s) are also done outside the laboratory.

NOTE Accidentally freezing the tissue (e.g. due to incorrect use of cool packs) can lead to biomolecule degradation when the tissue thaws thereafter. It can also impact the morphological characterization.

d) the labelling of the specimen container (e.g. patient identity, registration or hospital admission number, specimen type, specimen quantity, and organ tissue of origin) and additional documentation information as specified in 5.1.2, 5.1.3, and 5.1.4., a) to c)]. Two patient identifiers should be regarded as the minimum amount of information needed for proper patient/specimen donor identification. The specimen container labelling [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.

Specimens should be transferred without delay into the specimen container after the removal from the body. The specimen container then should be kept cold at 2 °C to 8 °C in order to minimize changes of morphology and/or biomolecules.

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 specimen container/ container compartment.

Temperature monitoring and documentation of the specimen containers' surrounding [e.g. temperatures during intermediate storage in different rooms; during transport (see 5.2)] should be performed in a suitable manner (e.g. temperature tracking). If the temperature cannot be measured, the temperature should be estimated and documented (e.g. as ambient temperature, room temperature, cold at 2 °C to 8 °C).

5.2 Transport

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

NOTE There is evidence that tissue morphology and biomolecules can be stabilized in plastic bags under vacuum when kept at 4 °C during transport[18] before the specimens are archived for biobanks or used for histopathological evaluation.

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

Temperature monitoring and recording should be applied in a suitable manner (e.g. by a temperature logger) (see 5.1.4).

Any deviations from the instructions shall be described and documented.

6 Activities inside the laboratory

6.1 Specimen reception

The identity of the person receiving the specimen shall be documented (e.g. in form of the name or a code).

The specimen arrival date and time shall be documented, and the 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 checked and documented.

Any deviations, including those from the established transport procedure (see 5.2), shall be documented. A procedure for handling nonconformities shall be in place. See also ISO 15189.

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 1 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 or before use (when not used and checked for over a week), and with every new batch.

NOTE 2 Formalin solution is not stable (e.g. it has a tendency to oxidize to formic acid)[19].

NOTE 3 The use of old formalin solution leads to formation of formic acid which lowers the pH value and can result in formalin pigment, which are black to brown amorphous to microcrystalline granules in histologic sections produced by acid acting upon haemoglobin[20].

The following steps shall be performed:

a) The documentation of the time point of placing the tissue specimen or sample into standard buffered formalin solution [to allow for calculation of the total fixation duration, which ends with transfer of the sample into the dehydration reagent during tissue processing (see 6.6)];

NOTE 1 The total formalin fixation duration can have an impact on further examinations, e.g. immunohistochemical techniques,[21] nucleic acid based molecular examinations.[22,23] The optimal formalin fixation duration can vary depending on tissue type and size.[48] For larger surgical specimens, e.g. a resected liver, inhomogeneous fixation can occur before the grossing process due to penetration of formaldehyde from the surface of the tissue to the interior.

NOTE 2 Formalin fixation of surgical specimens for more than 24 h can lead to a crosslinking intensity that can impact in situ examinations (e.g. immunohistochemistry) depending on the epitope.[24] Antigen retrieval can in many cases reverse these effects[17] but extensive antigen retrieval can also lead to artefacts (e.g. non-specific, false positive staining; impaired morphology).

NOTE 3 Too short formalin fixation (under fixation) results in a mixture of formaldehyde and alcohol fixation because formalin fixation starts at the tissue margin; in the unfixed tissue center fixation by alcohol (coagulation) occurs during tissue processing[25].

NOTE 4 The manufacturer's safety data sheet (MSDS) contains important information for handling standard buffered formalin solution.

NOTE 5 Formaldehyde is a carcinogenic and hazardous compound that penetrates the tissue and chemically modifies biomolecules. However, there are potential different local classifications.

b) the selection and use of container(s) for fixation:

   1) the capacity of the containers used for specimen/sample fixation should be such that the specimen can be completely submerged into the standard buffered formalin solution and the minimum standard buffered formalin solution to tissue ratio should be at least 10:1 (volume to volume)[10]. Where the original specimen container does not have the capacity required for a 10:1 volume to volume ratio, the specimen should be transferred to a larger container to achieve this ratio. Labelling of the larger container shall be identical to labelling of the original specimen container. Labelling can be complemented with the unique laboratory specimen identification number (pathology case number).

      NOTE 6 For in situ RNA examinations a ratio of 15:1 to 20:1 (volume to volume) is typically used[47].

      NOTE 7 There is evidence that lower formalin to tissue ratios (e.g. 2:1 or 1:1) can be used in combination with appropriate devices (e.g. under vacuum fixation) or if appropriate grossing is performed[26-28].

   2) to ensure complete formalin fixation of larger specimens, special tissue handling such as incision(s) (e.g. bisectioning) of solid organs or opening of hollow organs should be performed. In this case, the standard buffered formalin solution shall be changed periodically.

   3) when using specimen containers pre-filled with standard buffered formalin solution, the manufacturer's instructions shall be followed;

   4) the specimen container used for fixation shall be securely closable;

c) the composition of the formalin solution, the formalin fixation method (e.g. immersion fixation, or ultrasound of tissue fixation) and conditions (e.g. duration, temperature) used shall be documented.

   NOTE 8 Conventional fixation by immersion traditionally occurs at ambient temperature. There is evidence of superior immunohistochemistry staining after formalin injection, perfusion and ultrasound[24,29,30].

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

6.3.1 General

Local, national or regional regulations can apply.

Where the tissue specimen was removed from the body with the requirement of a histopathological diagnosis, the macroscopic 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.

NOTE Molecular features in tissues, particularly under disease conditions, are usually not homogeneously distributed. For example, different molecular mechanisms can be activated in the centre and at the invasion front of a tumor, also tumors can be composed of areas showing different differentiation grades.

6.3.2 Macroscopic evaluation

In the context of the macroscopic evaluation of the surgical specimen before and/or after formalin fixation, the patient/donor identity (e.g. the name or code), date of birth of the patient/speciment donor, hospital admission number and/or pathology case number and the clinical information (see 5.1.2 and 5.1.3) of the specimen (e.g. type, size, number) should be checked.

The surgical specimen and all macroscopic 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 anatomical localization represented in the specimen shall be described. Resection margins and other important areas may be marked, where necessary and helpful for later microscopic evaluation. This shall be documented.

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

6.3.3 Selection of the sample(s)

The selection of appropriate parts of the specimen for in situ examinations of morphology and biomolecules as well as for further research purposes shall be done by or under supervision of a medically qualified (e.g. board certified) pathologist. Representative samples for in situ examination shall be taken (i.e. grossing) according to the organ/disease specific guidelines from the respective medical societies and documented. In addition, for certain in situ detection techniques (e.g. immunohistochemistry) (a) sample(s) from the tumor border containing tumor and adjacent non-tumor tissue should be collected, where possible.

NOTE 1 Depending on local procedures, the selection of appropriate parts of the specimen can also be done by pathologists outside of the laboratory, e.g. in the operating theatre (see 5.1.3).

Where the tissue specimen was removed from the body without the requirement of a histopathological diagnosis, the documentation of this specimen, and the evaluation, selection, and documentation of the samples shall be done, but may be done by other qualified persons than pathologists.

The documentation can include photographs and schematic drawings.

The size of the selected sample(s) shall be appropriate for the tissue cassette (maximum of approximately 3 cm × 2 cm × 0,5 cm).

Where the specimen and the selected samples are not yet formalin-fixed, they shall be placed without delay into standard buffered formalin solution and all steps shall be performed as described in 6.2.

Where the specimen has already been placed into formalin outside the laboratory (see 6.2), the selected samples shall be transferred into a tissue cassette and, without delay, into standard buffered formalin solution to avoid drying of the sample. If the selected sample is not yet fixed appropriately, post-fixation in formalin should be performed. This can be performed within the tissue cassette.

The time point when the sample taken from the specimen is transferred into the tissue cassettes shall be documented. Instructions for the orientation of one or multiple samples (e.g. lymph nodes) in a tissue cassette shall be in place and followed.

NOTE 2 For in situ detection, the orientation of the sample in the tissue cassette and in the embedding mold determines which part of the sample is sectioned first. Typically, the tissue surface of interest is placed down in the cassette for fixation and this orientation is preserved during embedding when placing the tissue in the embedding mold. If multiple tissues (e.g. sentinel lymph nodes from different levels) are placed into one cassette for fixation and into the mold for embedding, the positions are typically relevant for histopathological diagnosis.

Each tissue cassette shall be labelled with a unique identifier (e.g. pathology case number, cassette number, tissue abbreviation). The identifier shall be indelible throughout all subsequent procedures. The unique identifier shall be human readable and can be complemented by a barcode, which can be applied manually or electronically by automated printers.

Minimum requirements for a unique identifier shall include: Laboratory specimen identification number (pathology case number) - including year, subsection type (surgical, cytology etc.); Specimen identifier – alpha or numeric; Block identifier – alpha or numeric.

Additional identifiers can be used (e.g. laboratory name or identifier, color coded cassettes for tissue type, fixative used, and/or pathologist etc.).

Where 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.

6.4 Post-fixation of frozen samples from intraoperative consultation

Where required, frozen specimens or samples (e.g. after frozen section diagnosis) may be post-fixed. This shall be performed in standard buffered formalin solution for further paraffin embedding as described in 6.2.

Where an FFPE specimen or sample was generated from a frozen specimen or sample, this shall be documented.

Residual tissues used for intraoperative examination shall be processed into paraffin for comparison with the frozen section interpretation.

6.5 Sample decalcification/softening

Some samples such as bone, bone-containing, or other calcified (e.g. tumors) or hard (e.g. keratinized) samples can require decalcification or softening to obtain FFPE tissue sections of adequate quality for in situ detection techniques.

Where decalcification and/or softening are performed, this shall be done according to manufacturer instructions or written instructions based on verified and validated procedures. Instructions should include, but are not limited to, the type and concentration of decalcifier/softening reagents, and the temperature and duration of decalcification/softening. It shall be documented which samples were subjected to decalcification and/or softening.

NOTE 1 There is evidence that decalcification procedures can affect examinations using in situ detection[24,31-33]. Different decalcification methods (e.g. incubation in oven/water bath, or ultrasound-acceleration) using different decalcifying reagents [such as strong inorganic (e.g. hydrochloric acid) or weak organic acids (e.g. formic acid)] can affect examinations by in situ detection techniques differently[24,30]. Decalcification is performed after complete formalin fixation and before tissue processing and embedding.

Where decalcification is performed following formalin fixation, the time point and date when the tissue is transferred from the formalin solution to the first reagent used in the decalcification procedure (e.g. decalcifier or washing solution) shall be documented to allow for calculation of the total fixation duration.

Where the specimen is transferred into formalin again after decalcification, this time point shall also be documented as fixation continues thereafter.

NOTE 2 Some decalcifier reagents can require the formalin washed from the specimen before transfer into the decalcifier and some before transfer into the dehydration solution (because e.g. placing EDTA decalcified tissue samples directly into 70 % (by volume) alcohol can lead to EDTA precipitations)[34].

6.6 Tissue processing and paraffin embedding

6.6.1 General

Tissue processing shall include the steps dehydration, clearing, and impregnation with paraffin, and shall be followed by paraffin embedding. There are some validated protocols that do not require the clearing step.

NOTE 1 Tissue processing and paraffin embedding can impact the morphology and biomolecule integrity in fixed tissue. Particularly inadequate tissue processing (e.g. insufficient dehydration, incomplete impregnation with paraffin) can lead to tissue artefacts and impair in situ detection (e.g. weak staining, loss of antigen due to degradation, nonspecific reactivity[24]).

Tissue processing should be performed in an automated tissue processor. Where tissue processing is performed manually (and this is not in contradiction with the in situ examination manufacturer's instructions), the laboratory's own verified and validated procedures shall be followed.

Written procedures for tissue processing and paraffin embedding shall be in place, specifying the method (e.g. manually or automated), instruments (e.g. type of tissue processor and embedding center), reagents (e.g. type of dehydration reagent, paraffin), and steps/program (sequence, concentration, duration and temperature of reagents). The procedures shall be according to the in situ examination manufacturer's instructions or to the laboratorie's verified and validated procedures where there are no manufacturers' instructions or other justified reasons. The applied procedure shall be documented. An example protocol is given in Annex B.

The time point and date when the specimen or sample is transferred from the formalin into the dehydration reagent (e.g. alcohol-containing solution) shall be documented to allow for calculation of the total fixation duration.

The replacement of all reagents shall be done on a regular basis, for automated tissue processing according to the tissue processor manufacturer's instructions. The replacement shall be documented (including date of replacement and type of reagent(s) replaced).

NOTE 2 Dehydrating and clearing solutions in tissue processing become diluted with carry-over from prior steps resulting in less efficient removal of water from the tissue. Residual water in the tissue cannot be replaced by paraffin and makes the tissue susceptible to degradation, particularly during storage.[10,35] Replacement intervals depend e.g. on the number of tissue cassettes processed and the date of the last replacement.

NOTE 3 Tissue processors exist, which have automated reagent management systems to measure and indicate to the user reached threshold concentration values of reagents; other tissue processors employ raised temperatures and/or use microwave energy to reduce processing times.

6.6.2 Dehydration and clearing

For dehydration, a series of dehydrating reagent (e.g. ethanol) solutions of increasing concentration up to water free (100 %) reagent shall be used (see Annex B).

NOTE 1 During tissue processing, the tissue is dehydrated, and water replaced with dehydrating reagent, most commonly ethanol. Residual water due to insufficient dehydration and clearing can affect the quality and stability of tissues, including biomolecules, during storage[10,13,25,35].

NOTE 2 Different alcoholic and non-alcoholic dehydration reagents (e.g. ethanol, isopropanol, methanol, glycol and denatured alcohols) exist and can differently impact the in situ examination[24,36].

NOTE 3 Excessive dehydration causes tissue shrinking and renders the tissue hard and brittle. Prolonged exposure to most clearing reagents causes the tissue to become brittle.[36] Fast dehydration (e.g. without increasing concentrations of dehydration reagent solutions) can increase tissue/cell distortion.

6.6.3 Impregnation with paraffin and paraffin embedding

The type of paraffin, the durations and temperatures of impregnation and paraffin embedding can have an influence on the quality of biomolecules. Paraffin with standardized composition and with low melting temperature should be used.

NOTE 1 Typical low melting point temperatures for paraffin are in the range from 50 °C to 56 °C. There is evidence that high-melting-point (65 °C) polymer paraffin can impact in situ detection examinations (e.g. IHC) [24].

The written procedures for paraffin embedding (see 6.6.1) shall include instructions for the correct orientation of the specimen/sample in the tissue cassette.

NOTE 2 Incorrect orientation of specimens/samples in tissue cassettes can result in diagnostically important tissue elements being missed or damaged during sectioning. It can also affect the ease of sectioning and thus tissue section quality.

NOTE 3 Special orientation can be required for some tissues (e.g. tubular structures such as blood vessels to allow for cutting in cross section of the lumen; tissues with an epithelial surface such as skin or intestine to allow for cutting of all relevant layers and to avoid compression of the tissue during sectioning).

NOTE 4 Products (e.g. gels, sponges, pads, papers) are available that help stabilize the position and orientation of a specimen/sample in the tissue cassette during tissue processing and embedding.

The tissue cassette containing the embedded tissues shall be cooled rapidly by an adequate means (e.g. cold plate for embedding).

NOTE 5 Rapid cooling of paraffin ensures formation of small crystalline structures, producing fewer artefacts when sectioning FFPE tissue[36].

6.7 Storage of FFPE tissue blocks

After complete cooling of embedded formalin-fixed tissue, the FFPE tissue blocks can be either directly subjected to sectioning (see 6.8) for subsequent in situ detection techniques or stored.

In general, storage of FFPE tissue can occur in several ways, e.g. as FFPE tissue blocks or cut (slide-mounted, unstained) sections (see 6.8)[37].

Storage time and condition (e.g. humidity, temperature, exposure to oxygen) of FFPE tissue blocks can have an impact over time on in situ detection examination result[13,37,38]. While morphology is hardly affected by storage, biomolecule integrity, immunoreactivity and nucleic acid in situ hybridization signals can decrease with increasing storage time, especially if FFPE tissue blocks are stored for many years[13,24,38].

In order to minimize changes in biomolecule integrity, immunoreactivity and nucleic acid in situ hybridization signals, FFPE tissue blocks should be stored dry at room temperature or preferably at lower temperature[46].

NOTE 1 Lower storage temperatures (e.g. 2 °C to 8 °C, –20 °C) slow down the RNA,[39,40,46] DNA and protein degradation processes, and the loss of immunoreactivity over time.

NOTE 2 If FFPE tissue is not stored dry and/or tissue processing is insufficient, biomolecule degradation and loss of immunoreactivity can increase, and fungal and bacterial growth can be stimulated.

NOTE 3 Covering of cut FFPE blocks with paraffin can be used for long-term storage.

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

6.8 Sectioning of FFPE tissue blocks and storage of slide-mounted sections

For immunohistochemistry and in situ hybridization, FFPE sections should be freshly cut. Where storage of slide-mounted sections cannot be avoided, they should be stored dry and at a temperature of 4 °C or lower temperature for as short as possible. The total storage duration limit specified in the in situ examination manufacturer's instructions or the laboratory's own instructions shall not be exceeded. The applied storage conditions (e.g. humidity, temperature, measures to prevent exposure to oxygen, such as storage under nitrogen, or coverage with paraffin) shall be documented. The start of storage (i.e. the date of sectioning/slide-mounting) shall be documented to allow for calculation of the total storage duration.

NOTE 1 Exposure of the section to air can result in oxidation of biomolecules, which can lead to antigen alteration.

NOTE 2 Unstained sections are much more susceptible to biomolecular degradation during storage than FFPE blocks. Storage of slide-mounted unstained FFPE sections can impact the immunohistochemical staining of antigens. Not all antigens are equally affected by antigen decay. For many antigens, a section storage time of 1 month to 2 months is without adverse effects. For some antigens, detection is impaired already after 1 week to 3 weeks of storage[24].

The type of slides (e.g. charge, coating with adhesive), FFPE tissue section thickness, drying (e.g. duration, temperature, method) and storage (e.g. duration, condition) of unstained slide-mounted sections can each impact the outcome of an in situ detection[24,30] and shall be according to the in situ examination manufacturer's instructions. Where no in situ examination manufacturer's instructions are provided or not all of these factors are addressed, the laboratory shall verify and validate a procedure, and provide and follow the written instructions.

If sections are cut for external parties performing the in situ detection, issues such as the type and adhesive of the slide, the thickness of the section or the susceptibility of the in situ detection method to storage time of the section, shall be clarified in advance.

Slides shall be clearly labelled with the information from the tissue cassette complemented by information on the stain/detection system if applicable. If multiple sections are cut from one FFPE block and mounted onto different slides, the sequential order of sections should be included in the label (i.e. slide level number). Only sections from one patient shall be placed on a slide. This does not apply for control sections.

Labelling shall be compatible with the intended in situ examination (e.g. withstand reagents and temperatures used during the in situ examination). Where digitalization of stained sections is intended, the slide labelling shall be human and machine-readable (e.g. bar-code). Labelling should be performed at the time of sectioning and shall be matched to the label of the corresponding FFPE tissue block.

For RNA in situ detection techniques, devices (e.g. microtome blade, slides) and reagents (water in the flotation bath) that come into contact with the tissue section should be RNase-free.

FFPE tissue blocks shall be trimmed before taking the sections.

The thickness of sections for in situ detection shall be as specified in the in situ examination manufacturer's or the laboratory's own instructions.

NOTE 3 Typically, sections of 2 µm to 5 µm thickness are used. Some in situ detection techniques and/or types of tissues can require sections of other thicknesses. Factors such as FFPE tissue block temperature, speed of rotation or clearance angle setting can influence the actual thickness achieved.

Maintenance and/or services of microtomes should be performed on a regular basis and documented (including but not limited to the instrument serviced and the date of service) (see ISO 15189).

Water temperature of the flotation (water) bath should be 5 °C to 10 °C below the melting point of the paraffin and over-heating avoided. The temperature before use shall be documented. The flotation (water) bath shall be cleaned after each block (e.g. using a tissue paper) to remove tissue fragments and debris.

Where applied, soaking of FFPE blocks in solutions (e.g. for surface decalcification or tissue softening) that can impact in situ detection shall be documented.

Drying temperature, duration time and method (e.g. oven drying, (forced) air-drying, drying on-board automated stainers) of the slide-mounted FFPE tissue sections shall be documented.

NOTE 4 Drying temperature and duration can vary depending on the type of tissue. Recommended drying time/temperature for in situ detection techniques is 1 h at 60° C. Temperatures of above 68° C have been shown to diminish IHC staining intensity,[24] lead to morphological (e.g. nuclear) alterations, or loss of biomolecules (e.g. fat)[41].

6.9 Deparaffinization and rehydration of slide-mounted sections

The slide-mounted tissue sections shall be deparaffinized shortly prior to in situ detection or, where pretreatment is necessary, prior to pretreatment (see 6.10) (e.g. antigen retrieval for IHC or pre-hybridization for ISH). Depending on the analytical in situ test, rehydration of deparaffinized sections can be required (e.g. staining with aqueous histological stains or incubation with antibodies) or omitted (e.g. with certain in situ hybridization tests). Where rehydration is required, this shall be done directly after deparaffinization.

Where in situ examination manufacturer's instructions for deparaffinization and rehydration are provided, these shall be followed.

Where no in situ examination manufacturer's instructions for deparaffinization and rehydration are provided, the laboratory shall develop and verify its own deparaffinization and rehydration procedures according to the examination performance specifications. This shall include but is not limited to the types of reagents, the method (e.g. manual, automated), device (e.g. oven, automated stainer), durations and temperatures. Written and where needed visual instructions shall be provided and followed.

NOTE 1 Automated stainers exist with on-board functions for drying, deparaffinization and rehydration of slide-mounted sections.

NOTE 2 Incomplete deparaffinization [42] and high temperatures can impact certain in situ detection techniques.

6.10 Pretreatment of slide-mounted sections for in situ detection techniques

6.10.1 General

For some in situ detection techniques and analytical tests, pretreatment of slide-mounted deparaffinized and rehydrated FFPE tissue sections can be required to reverse modifications from formalin fixation, tissue processing and embedding (e.g. to unmask antigens/epitopes and target nucleic acids). Pretreatment is often integrated in the in situ detection technique.

6.10.2 Pretreatment for antibody- or other affinity binder-based in situ detection techniques

For in situ detection techniques based on antibodies or other affinity binders (e.g. immunohistochemistry, immunofluorescence staining), antigen retrieval (e.g. heat-induced epitope retrieval/HIER) can be required as pretreatment.

Where in situ examination manufacturer's instructions on antigen retrieval are provided, these shall be followed.

Where no in situ examination manufacturer's instructions on antigen retrieval are provided, the examination laboratory shall design, specify, develop, verify and validate the procedures. This shall

include but is not limited to the type of antigen retrieval (e.g. heat-induced or proteolytic-induced epitope retrieval), devices (e.g. automated stainer when performed on board, microwave, water bath, pressure cooker), as well as antigen retrieval reagents (e.g. type and pH of buffers, type and concentration of enzyme), duration and condition (e.g. duration, temperature, watt).[3] Instructions for the pretreatment procedure shall be established for each analytical test and followed.

The applied pretreatment procedure shall be documented.

6.10.3 Pretreatment for hybridization-based in situ detection techniques

For hybridization-based in situ detection techniques (e.g. fluorescence or chromogenic in situ hybridization), permeabilization of the tissue and cells, target unmasking and blocking (also referred to as pre-hybridization) can be required as pretreatment.

Where in situ examination manufacturer's instructions on pretreatment are provided, these shall be followed.

Where no in situ examination manufacturer's instructions on pretreatment are provided, the examination laboratory shall design, specify, develop, verify and validate the procedure. This shall include but is not limited to the pretreatment reagents (e.g. type and concentration of enzymes and buffers), steps, durations, temperatures and devices. Instructions for the pretreatment procedure shall be established for each analytical test and followed.

The applied pretreatment procedure shall be documented.

6.10.4 Pretreatment for other in situ detection techniques

In general, there is no pretreatment before classical histological staining and histochemical techniques.

Other in situ detection methods such as molecular analyses of isolated biomolecules that can be mapped to a defined region of an FFPE section (by e.g. in situ sequencing, imaging mass spectrometry) can require special pretreatment of the tissue section.

6.11 Quality assessment of the pre-analytical part of in situ detection

Quality assessment shall follow the examination manufacturer's instructions. Where these do not contain information on quality assessment, the laboratory shall develop and verify the quality assessment procedure. Quality assessment can include but is not limited to assessment of the quality of the histological sections (e.g. section thickness, distortions, folds, cellular morphology including chromatin appearance) and the quality and integrity of biomolecules. Quality and integrity of biomolecules can be assessed by using antibodies to antigens that are sensitive to pre-analytical procedures or by quality assessment of biomolecules isolated from the sections (e.g. integrity of RNA isolated from the sections).

Certain structures within the tissue section can serve as internal positive and negative controls for each in situ detection test. For immunohistochemistry, positive control sections shall be placed on the same slide as the patient section. Analysis of digitized in situ detection images (e.g. generated by using a slide scanner) can require more stringent pre-analytical quality criteria than analysis by microscope. Where in situ detection on FFPE tissue is performed and the slides are digitized for analysis by machine learning algorithms on digital images, even more stringent pre-analytical quality criteria can be required.

For example, pathologists recognize fixation or cutting artefacts and consider these properly in their diagnoses, whereas results generated by machine learning algorithms can be strongly affected, which can lead to wrong assessment[49].

Annex A
(informative)

Recommendations relating to verification and validation of laboratory developed in situ detection tests

Cases used for validation should reflect the same pre-examination processes as cases tested clinically.

Examples of analyses to be performed for validating pre examination procedures of an in situ detection test developed in the laboratory:

1. Correlating the new test's results with the morphology and expected results;
2. Comparing the new test's results with the results of prior testing of the same tissues with a validated assay in the same laboratory;
3. Comparing the new test's results with the results of testing the same tissue validation set in another laboratory using a validated assay;
4. Comparing the new test's results with previously validated non-immunohistochemical tests; or
5. Testing previously graded tissue challenges from a formal proficiency testing program (if available) and comparing the results with the graded responses[43,44].