
**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

ISO 20166-4:2021

<https://standards.iteh.ai/catalog/standards/iso/49d8dbc8-701c-4218-bd74-567cf1212065/iso-20166-4-2021>



iTeh Standards
(<https://standards.iteh.ai>)
Document Preview

ISO 20166-4:2021

<https://standards.iteh.ai/catalog/standards/iso/49d8dbc8-701c-4218-bd74-567cf1212065/iso-20166-4-2021>



COPYRIGHT PROTECTED DOCUMENT

© ISO 2021

All rights reserved. Unless otherwise specified, or required in the context of its implementation, no part of this publication may be reproduced or utilized otherwise in any form or by any means, electronic or mechanical, including photocopying, or posting on the internet or an intranet, without prior written permission. Permission can be requested from either ISO at the address below or ISO's member body in the country of the requester.

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

Published in Switzerland

Contents

Page

Foreword	iv
Introduction	v
1 Scope	1
2 Normative references	1
3 Terms and definitions	1
4 General considerations	8
5 Activities outside the laboratory	10
5.1 Specimen collection.....	10
5.1.1 General.....	10
5.1.2 Information about the patient/specimen donor.....	10
5.1.3 Information about the specimen.....	10
5.1.4 Specimen processing, intermediate storage and preparation for transport.....	11
5.2 Transport.....	12
6 Activities inside the laboratory	12
6.1 Specimen reception.....	12
6.2 Formalin fixation of the specimen or sample(s).....	12
6.3 Evaluation of the pathology of the specimen and selection of the sample(s).....	13
6.3.1 General.....	13
6.3.2 Macroscopic evaluation.....	14
6.3.3 Selection of the sample(s).....	14
6.4 Post-fixation of frozen samples from intraoperative consultation.....	15
6.5 Sample decalcification/softening.....	15
6.6 Tissue processing and paraffin embedding.....	16
6.6.1 General.....	16
6.6.2 Dehydration and clearing.....	16
6.6.3 Impregnation with paraffin and paraffin embedding.....	17
6.7 Storage of FFPE tissue blocks.....	17
6.8 Sectioning of FFPE tissue blocks and storage of slide-mounted sections.....	18
6.9 Deparaffinization and rehydration of slide-mounted sections.....	19
6.10 Pretreatment of slide-mounted sections for in situ detection techniques.....	19
6.10.1 General.....	19
6.10.2 Pretreatment for antibody- or other affinity binder-based in situ detection techniques.....	19
6.10.3 Pretreatment for hybridization-based in situ detection techniques.....	20
6.10.4 Pretreatment for other in situ detection techniques.....	20
6.11 Quality assessment of the pre-analytical part of in situ detection.....	20
Annex A (informative) Recommendations relating to verification and validation of laboratory developed in situ detection tests	21
Annex B (informative) Example protocol for tissue processing	22
Bibliography	23

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.

iTeh Standards
(<https://standards.itih.ai>)
Document Preview

[ISO 20166-4:2021](https://standards.itih.ai/catalog/standards/iso/49d8dbc8-701c-4218-bd74-567cf1212065/iso-20166-4-2021)

<https://standards.itih.ai/catalog/standards/iso/49d8dbc8-701c-4218-bd74-567cf1212065/iso-20166-4-2021>

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

ISO 20166-4:2021
<https://www.iso.org/standard/4948883.html> [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