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

Part 2: Isolated proteins

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

Partie 2: Protéines extraites

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Foreword

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

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

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*.

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.

A list of all parts in the ISO 20166 series can be found on the ISO website.

Introduction

Molecular in vitro diagnostics, including molecular pathology, has enabled a significant progress in medicine. Further progress is expected with new technologies analyzing 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.

Although originally thought as being impossible due to the crosslinking activities of formaldehyde, protein isolation techniques from formalin-fixed and paraffin-embedded (FFPE) tissues have been much improved in recent years. Heat-induced reversal of formaldehyde-induced crosslinks has been demonstrated as an essential step in the protein isolation procedures[5][6]. Currently, most investigators accept that proteins isolated from FFPE tissue are suitable for downstream proteomic examination[7].

Protein profiles, protein integrities, and protein–protein interactions in tissues can change drastically before, during and after collection (due to, e.g. gene induction, gene down regulation, protein degradation). Protein species amounts can change differently in different donors'/patients' tissues. The expression of genes can be influenced by the given treatment or intervention (surgery, biopsy), or drugs administered for anaesthesia or even treatment of concomitant disease as well as by the different environmental conditions after the tissue removal from the body.

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

Therefore, it is essential to take special measures to minimize the described protein profile changes and modifications within tissues for subsequent examination.

A standardization of the entire process from specimen collection to the protein examination is needed. Studies have been undertaken to determine the important influencing factors. This document draws upon such work to codify and standardize the steps for FFPE tissue with regard to protein examination in what is referred to as the pre-examination phase.

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

Part 2: Isolated proteins

1 Scope

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

This document is not applicable for protein examination by immunohistochemistry.

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

2 Normative references

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

ISO 15189:2012, *Medical laboratories — Requirements for quality and competence*

ISO 15190, *Medical laboratories — Requirements for safety*

ISO/IEC 17020:2012, *Conformity assessment — Requirements for the operation of various types of bodies performing inspection*

3 Terms and definitions

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
aliquot
portion of a larger amount of homogeneous material, assumed to be taken with negligible sampling error

Note 1 to entry: The term is usually applied to fluids. Tissues are heterogeneous and therefore cannot be aliquoted.

Note 2 to entry: The definition is derived from References [28], [29], and [30].

3.2
ambient temperature
unregulated temperature of the surrounding air

3.3
analyte
component represented in the name of a measurable quantity

[SOURCE: ISO 17511:2003, 3.2 — EXAMPLE has been removed.]

3.4
analytical test performance
accuracy, precision, and sensitivity of a test to measure the *analyte* (3.3) of interest

Note 1 to entry: Other test performance characteristics such as robustness, repeatability can apply as well.

3.5
cold ischemia
condition after removal of the tissue from the body until stabilization or fixation

3.6
diagnosis
identification of a health or disease state from its signs and/or symptoms, where the diagnostic process can involve *examinations* (3.7) 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
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.]

3.8
formalin
saturated aqueous formaldehyde solution which at 100 % contains 37 % formaldehyde by mass (corresponding to 40 % by volume)

3.9
formalin fixation
treatment of a sample with *standard buffered formalin solution* (3.21) for stabilization

3.10
grossing
gross examination
inspection of pathology specimens with the bare eye to obtain diagnostic information, while being processed for further microscopic examination

3.11
paraffin embedding
process in which a tissue *sample* (3.19) is placed in paraffin to achieve a hard surrounding matrix so that thin microscopic sections can be cut

3.12
pre-examination process
pre-analytical phase
pre-analytical workflow
process that starts, in chronological order, from the clinician's request and includes the examination request, preparation and identification of the patient, collection of the primary sample(s), transportation to and within the medical or pathology laboratory, isolation of analytes, and ends when the analytical examination begins

Note 1 to entry: The pre-examination phase includes preparative processes, e.g. protein isolation procedures, which 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.]

3.13
primary sample
specimen
discrete portion of a body fluid, breath, hair or tissue taken for examination (3.7), 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.]

3.14
protein
type of biological macromolecule composed of one or more chains with a defined sequence of amino acids connected through peptide bonds

3.15
protein profile
amounts of the individual *protein* (3.14) molecules that are present in a sample and that can be measured in the absence of any losses, inhibition and interference

3.16
protein species
amounts of a chemically clearly-defined protein corresponding to one spot on a high-performance two-dimensional gel electrophoresis pattern

Note 1 to entry: The definition is taken from Reference [7].

3.17
post-translational modification
chemical alterations to a primary protein structure, often crucial for conferring biological activity on a protein

Note 1 to entry: The definition is taken from Reference [8].

3.18
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.

3.19
sample
one or more parts taken from a *primary sample* (3.13)

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

3.20
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 protein.

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

3.21
standard buffered formalin solution
neutral buffered formalin
NBF
10 % *formalin* (3.8) solution in water with a mass fraction of 3,7 % (corresponding to a volume fraction of 4 %) formaldehyde, buffered to pH 6,8 to pH 7,2

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

3.22
storage
prolonged interruption of the *pre-analytical workflow* (3.12) of a sample or analyte 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.

3.23
tissue processor
automated instrument where tissue fixation, dehydration, clearing and paraffin infiltration occurs

3.24
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.]

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

3.26
workflow
series of activities necessary to complete a task

3.27
homogeneous
uniform in structure and composition

4 General considerations

For general statements on medical laboratory quality management systems and in particular on specimen collection, reception, and handling (including avoidance of cross contaminations) see ISO 15189:2012, 4.2, 5.4.4, 5.4.6 or ISO/IEC 17020:2012, Clause 8 and 7.2. The requirements on laboratory equipment, reagents, and consumables in accordance with ISO 15189:2012, 5.3 shall be followed; ISO 15189:2012, 5.5.1.2 and 5.5.1.3, and ISO/IEC 17020:2012, 6.2 can also apply.

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

The stability of the specific protein(s) of interest and their post-translational modifications (if important for the assay) should be investigated throughout the complete pre-examination process prior to the development and implementation of an examination test (e.g. by performing a time course experiment or study; see also Annex A and Reference [9]).

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

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

NOTE Prolonged cold ischemia results in changes of protein (e.g. cytokeratin 18) and phosphoprotein (e.g. phospho-p42/44) amounts[9][10]. Keeping the specimen on wet-ice diminishes this effect[11]. Protein amounts as well as the protein modifications 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.

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

In addition, the formalin fixation itself, as well as the subsequent FFPE tissue storage duration and storage temperature causes modifications of biomolecules and leads to suboptimal performance of protein isolated from FFPE tissues[12]. This should be considered in the quality control and application of molecular analytical tests. Analytical test optimization for FFPE tissues or the use of non-crosslinking alternatives to standard buffered formalin solution are options to minimize this issue for molecular examinations.

Safety instructions on transport and handling shall be considered and followed in accordance with ISO 15189:2012, 5.2.3 and 5.4.5, and ISO 15190.

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

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

5 Outside the laboratory

5.1 Specimen collection

5.1.1 General

For the collection of the specimen, the requirements (e.g. disease condition, specimen size) for the intended molecular examination (see also Clause 6) should be considered.

See also ISO 15189:2012, 5.4.4.

5.1.2 Information about the specimen donor/patient

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

The documentation should include, but is not limited to:

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

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

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

5.1.3 Information about the specimen

The documentation shall include, but is not limited to:

a) the start of ischemia within the body (warm ischemia) by documentation of the ischemia-relevant vessel ligation/clamping time point (usually arterial clamping time);

b) the time and date when tissue is removed from the body and the method of removal (e.g. core-needle biopsy, resection, biopsy device used for the collection);

c) the description of tissue type and origin, tissue condition (e.g. diseased, unaffected by the disease), including references to any marking applied in or outside the operating theatre made by surgeon, radiologist or pathologist;

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

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

5.1.4 Specimen processing

The following steps shall be performed:

a) the documentation of any 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 protein degradation when the tissue thaws thereafter. It can also impact the morphological characterization.

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

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

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

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

The temperatures of the container's surroundings during cold ischemia (e.g. temperatures in different rooms; transport) should be documented. If the temperature cannot be measured, the temperature range should be estimated by classification as ambient temperature, room temperature, or at 2 °C to 8 °C.

5.2 Transport requirements

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

Temperature monitoring should be applied in a suitable manner.

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

NOTE There is evidence that proteins in tissues can be stabilized in plastic bags under vacuum when kept at 0 °C to 4 °C during transport[13] before the samples are archived for biobanks or used for histopathological evaluation.

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

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

6 Inside the laboratory

6.1 Information about the reception of the specimen

The ID or name of the person receiving the specimen shall be documented. The specimen arrival date and time, and conditions (e.g. labelling, transport conditions including temperature, tissue type and quantity of the specimen, leaking/breaking of the container) of the received specimens shall be documented. Any deviations from the established protocol for the transport procedure (see 5.2) shall be documented.

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

6.2 Formalin fixation of the specimen or sample(s)

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

The fixative used should be standard buffered formalin solution.

NOTE 1 In some countries, standard buffered formalin solution is referred to as neutral buffered formalin (NBF).

NOTE 2 There is evidence that the yield of certain proteins, as determined by Western blot examination, can be increased by ultrasound mediated acceleration of tissue fixation[14].

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 oxidize to formic acid)[15].

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 specimen or sample into standard buffered formalin solution;

NOTE The total formalin fixation duration can have an impact on further examinations, e.g. immunohistochemical techniques, nucleic acid based molecular examinations. 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. Formalin fixation for more than 24 h can lead to a crosslinking intensity that can impact the protein examination test. It has been shown that the protein yield decreased with increasing fixation duration[16][17].

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 6.7.2.

c) the selection of container(s):

1) the capacity of the 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)[18]. To ensure complete formalin fixation of larger specimens, a special tissue handling such as incision(s) of solid organs or opening of hollow organs should be performed.

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

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

3) the container shall be securely closable;

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

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

2) the basic specimen information, e.g. the tissue type, tissue condition, and related additional information such as affected (e.g. tumour) or unaffected, unless a sample tracking system can supply this information coupled to the identification of the specimen or sample used in 6.2 d) 1);

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

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

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

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

The evaluation and documentation of the pathology of the specimen and the selection of the sample(s) from the specimen for further processing shall be done by or under supervision or responsibility of a medically qualified (e.g. board certified) pathologist.

Local, national or regional regulations can apply.

Options to select the sample(s) for protein examination:

a) The selection of appropriate parts of the specimen for molecular and histopathological examinations as well as for 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 protein 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 5.1.3).

   In the context of the macroscopic evaluation of the surgical specimen before and/or after formalin fixation, the clinical information (see 5.1.2 and 5.1.3), of the specimen (e.g. type, size, number), hospital admission number and/or pathology case number and/or donor/patient ID, name of the patient, date of birth of the patient and type of tissue 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) Where the tissue specimen was removed from the body without the requirement of a histopathological diagnosis, the documentation of this specimen, the evaluation, selection and documentation of the samples may be done by other qualified persons than pathologists.

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

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

Without delay, 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.

In this case, the total formalin fixation duration period shall be documented.

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

6.5 Processing and paraffin embedding

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

NOTE 1 During processing, the tissue is dehydrated and water is replaced with paraffin wax. Residual water can affect the quality and stability of tissues, including protein, during storage[18].

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.

The paraffin and embedding procedures can have an influence on the quality of proteins especially the (high) temperature applied and the process duration. The protein examination test shall therefore be validated and verified for the paraffin embedding processes used. If impacts on the examination test are recognized, the temperature and/or the duration should be reduced.

6.6 Storage requirements

FFPE tissue can be stored in several ways, e.g. as blocks, cut sections or as multi tissue arrays[19]. Storage time may influence the retrieval of a protein or group of proteins over time and thus impact the subsequent proteomic measurements[20]. While histology is hardly affected by storage, protein yield may decrease with increasing storage time, especially if specimens or samples are stored for years[19].

Storage conditions, e.g. humidity and temperature, can have an impact on protein amounts in archival FFPE tissues[19].

NOTE 1 One study reported a decreased Western blot signal of proteins isolated from FFPE tissue sections depending on the storage temperature and humidity[21].

In order to minimize protein amount changes, FFPE tissue should be stored dry at room temperature or preferably at lower temperature.

NOTE 2 Lower storage temperatures (e.g. 2 °C to 8 °C, –20 °C) slow down the protein degradation process over time.

NOTE 3 If FFPE tissue is not stored dry, the protein degradation can increase, and fungal and bacterial growth can be stimulated.

For protein isolation, FFPE sections should be freshly prepared. If storage of these sections cannot be avoided before isolating total protein, they should be stored dry and at room temperature or lower temperature for as short as possible.

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.

6.7 Isolation of the total protein

6.7.1 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[22]). When the specimen or sample is used for molecular diagnosis, the fraction of target cells shall be evaluated prior to the protein 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.

6.7.2 General information for protein isolation procedures

There are several challenges, including selective or incomplete recovery of proteins, protein degradation and protein modifications, that shall be taken into account to interpret the results obtained from the examination test.

Requirements and recommendations:

a) The optimal fixation duration depends on the tissue type and thickness[19]. Prolonged tissue fixation can result in reduced protein yields 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 protein purification should be freshly cut sections, with a thickness of up to 10 µm, obtained from FFPE tissue blocks, manually[23] dissected samples, laser microdissected (LMD)[24][25] 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 protein isolation and quantification. Staining sections prior to protein isolation should not be performed, as staining can impair protein quality and performance in downstream applications[23].

If protein is extracted from archived tissue blocks, the blocks should be trimmed by disposing the first sections before taking the sections for protein isolation. 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 the total protein is a key step in the diagnostic workflow, which shall be especially focused on during the verification and validation of the entire workflow.

6.7.3 Using commercial kits

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

6.7.4 Using the laboratories' own protocols

If a commercial kit is not used in accordance with its intended use, but is validated fit for purpose as defined by the user, instructions shall be written and followed.

If the laboratory uses its own protocol independent from a commercial kit, the validation and verification demonstrating that it is fit for purpose shall be carried out, and instructions shall be written and followed

A strong detergent (most commonly used is sodium dodecyl sulfate, SDS) and exposure to high temperatures should be used for an efficient reversal of formaldehyde-based protein crosslinks needed for protein isolation from FFPE tissues.

The combination of products from different manufacturers can compromise results as the products may not be compatible. They should be used for diagnostic testing only if the components have been tested together and validated to work satisfactorily.

Protein isolation procedures for FFPE tissue sections, which are mounted directly onto a glass slide, should contain the following three steps:

a) Specimen or sample preparation

— Using a microtome, a suitable number of 5-µm- to 10-µm-thick sections should be cut and then mounted onto glass microscope slides.

NOTE For certain tissues and/or proteins, thicker sections can work as well.

b) Deparaffinization

— Routine deparaffinization and rehydration steps can be used (e.g. addition of a suitable paraffin wax solvent, twice for 10 min and 100 %, 90 % and 70 % ethanol for 5 min each).

NOTE 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.

c) Protein isolation

— The desired tissue area should be transferred into a reaction tube containing a suitable volume of a protein isolation buffer containing a strong detergent, e.g. sodium dodecyl sulfate. The tissue should be boiled for 20 min, followed by incubation at 80 °C for 2 h. The tissue should be centrifuged and the supernatant be transferred into a fresh reaction tube for quality assessment.

— Depending on the subsequent examination test (e.g. mass spectrometry) additional steps (e.g. generation of peptides) can be required after the removal of the detergent.

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

NOTE Partial solubilisation of FFPE tissues can lead to isolation bias and can affect the final assay result[6].

6.8 Quality assessment of isolated proteins

The protein quality and quantity should be checked by generally accepted physical, chemical or biochemical procedures (e.g. Western blot[26], Bradford assay[27]), and/or by suitable controls as part of the examination test.

Such procedures to determine the purity and integrity may include one or more of the following techniques, depending on the specific examination test:

a) Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS–PAGE) and Coomassie blue or silver staining;

b) capillary electrophoresis;

c) mass spectrometry;

d) Western blot (e.g. β-actin).

Determining total protein concentration may include one or more of the following techniques, depending on the specific examination test:

a) Bradford assay;

b) bicinchoninic acid (BCA) assay;

c) Lowry assay.

6.9 Storage of isolated total protein

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

If there is no information available from the protein isolation kit provider or if the laboratories' own validated total protein isolation procedures are used, the isolated proteins should be assayed immediately. Where the protein cannot be assayed immediately, the laboratory shall have verified procedures in place on how to store the isolated protein.

NOTE 1 Storage in solution on wet-ice for a short period of time (i.e. 2 h) can be appropriate in certain circumstances.

Storage for long-term purposes (i.e. for several years) should be at ≤–70 °C. Other validated methods for archiving can also be used.

For long-term storage, aliquots of the isolated protein should be generated to avoid repeated freezing and thawing. Avoid more than two freeze–thaw cycles. If lyophilized, proteins can be stored for several years at 4 °C or –20 °C.

NOTE 2 The protein stability is affected by numerous factors, including freeze/thaw cycles, pH, protein concentration, salt conditions and others. Optimal conditions for storing specific proteins can vary from protein to protein.

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

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

Annex A
(informative)

Examination of protein demonstrates changes of protein amounts during cold ischemia[1)]

A.1 Introduction

Phosphorylation and dephosphorylation are key mechanisms of intra- and intercellular signal transduction and reflect the activation status of a cell. The identification of specific phosphoprotein profiles is being used to develop targeted therapies against deregulated signalling pathways in cancer patients. However, knowledge of the impact of pre-examination variations, such as delayed time to formalin fixation, on protein and phosphoprotein changes in the specimen is very limited.

The results of this study give insights into the inter-patient variability as well as the fluctuations of protein and phosphoprotein profiles in clinical tissue samples during the pre-examination phase. Using human intestine and liver tissues as examples, the data of this experimental work, as described below, clearly show that there is a need to standardize the collection of FFPE tissues and the subsequent isolation and storage of proteins and phosphoproteins before the quantitative examination. This standardization process includes the documentation of warm and cold ischemia durations. While the results for warm ischemia are reported elsewhere (see further reading), the data shown here indicate that cold ischemia has an influence on protein profiles. Thus, there is a risk that due to variations in warm and cold ischemia durations and other pre-examination parameters, the examination assay may be unreliable and meaningful biomarkers for treatment of patients may be missed or interpreted wrongly.

A.2 Example

A.2.1 General

In a time course experiment, human intestine and liver tissues were used to assess the influence of prolonged cold ischemia on the amounts of proteins and phosphoproteins in the specimens before formalin fixation. The data revealed that the protein and phosphoprotein amounts changed before the tissues are stabilized by fixation with standard buffer formalin solution.

These changes varied between different patients and tissue types. For example, up-regulation of phospho-p42/44 mitogen activated protein kinase (MAPK) in intestine samples was seen in some patients but not in others. This pronounced inter-patient variability prevented recognition of general trends within a patient cohort for up- or down-regulation of most proteins. However, amounts of a few proteins, such as cytokeratin 18, were altered significantly from the individual baseline in most patients' post-resection samples. In contrast, amounts of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin were found to be stable during prolonged cold ischemia.

A.2.2 Experimental procedures

A.2.2.1 General

Human intestine and liver tissues were collected in different hospitals using the same workflow. The time between vessel ligation (t_1) and surgical resection (t_2) is defined as warm ischemia (1). The time between surgical resection and formalin fixation, typically the transport time (2) to the pathology

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