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Molecular in vitro diagnostic examinations — Specifications for pre-examination processes for frozen tissue —

Part 2: Isolated proteins

Analyses de diagnostic moléculaire in vitro — Spécifications relatives aux processus préanalytiques pour les tissus congelés —

Partie 2: Protéines extraites

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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 20184 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 a significant progress in medicine. Further progress is expected with new technologies analysing nucleic acids, proteins, and metabolites in human tissues and body fluids. However, the profiles and/or integrity of these molecules can change drastically during specimen collection, transport, storage, and processing thus making the outcome from diagnostics or research unreliable or even impossible because the subsequent examination assay will not determine the situation in the patient but an artificial molecular pattern generated during the pre-examination process. Therefore, 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 frozen tissue with regard to protein examination in what is referred to as the pre-examination phase.

Protein profiles and protein–protein interactions in tissues can change drastically before, during (e.g. due to warm ischemia) and after tissue collection (e.g. due to cold ischemia). The changes are caused by 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.

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

Tissues that have undergone chemical stabilization pre-treatment before freezing are not covered in this document. In addition this document is not applicable to protein examination by immunohistochemistry.

In this document, the following verbal forms are used:

— "shall" indicates a requirement;

— "should" indicates a recommendation;

— "may" indicates a permission;

— "can" indicates a possibility or a capability.

Molecular in vitro diagnostic examinations — Specifications for pre-examination processes for frozen tissue —

Part 2: Isolated proteins

1 Scope

This document gives guidelines on the handling, documentation, storage and processing of frozen 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 any molecular in vitro diagnostic examination performed by medical laboratories and molecular pathology laboratories that evaluate proteins isolated from frozen tissue. It is also intended to be used by laboratory customers, in vitro diagnostics developers and manufacturers, biobanks, institutions and commercial organisations performing biomedical research, and regulatory authorities.

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

2 Normative references

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

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

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 homogenous 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 the Compendium of Chemical Terminology Gold Book. International Union of Pure and Applied Chemistry. Version 2.3.3., 2014; the PAC, 1990,62,1193 (Nomenclature for sampling in analytical chemistry (Recommendations 1990)) p. 1206; and the PAC 1990, 62, 2167 [Glossary of atmospheric chemistry terms (Recommendations 1990)] p. 2173.

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]

3.4
analytical test performance
accuracy, precision, and sensitivity of a test to measure the analyte 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 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 — The term and definition is used here without the original notes.]

3.8
grossing
gross examination
inspection of pathology specimens with the bare eye to obtain diagnostic information, while being processed for further microscopic examination

3.9
homogeneous
uniform in structure and composition

3.10
pre-examination processes
preanalytical phase
preanalytical workflow
processes that start, in chronological order, from the clinician's request and include 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 end when the analytical examination begins

Note 1 to entry: The pre-examination phase includes preparative processes that influence the outcome of the intended examination.

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

3.11
primary sample
specimen
discrete portion of a body fluid, breath, hair or tissue taken for examination, 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.]

3.12
protein
type of biological macromolecules composed of one or more chains with a defined sequence of amino acids connected through peptide bonds

3.13
protein profile
amounts of the individual protein molecules that are present in a sample and that can be measured in the absence of any losses, inhibition and interference

3.14
protein species
amounts of a chemically clearly-defined protein corresponding to one spot on a high-performance two-dimensional gel electrophoresis pattern

[SOURCE: Jungblut *et. al.*1996]

3.15
PTM
post translational modifications
chemical alterations to a primary protein structure, often crucial for conferring biological activity on a protein

[SOURCE: Encyclopedia of Psychopharmacology, 2010]

3.16
room temperature
temperature which is defined as 18 °C to 25 °C

Note 1 to entry: Local or national regulations can have different definitions.

3.17
sample
one or more parts taken from a primary sample

[SOURCE: ISO 15189:2012, 3.24, modified — The example was not taken over.]

3.18
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

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

Note 1 to entry: The analyte for the purpose of this document is isolated protein.

3.19
storage
prolonged interruption of the pre-analytical workflow of a sample or analyte respectively, or of their derivatives e.g., 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.

3.20
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 term "validated" is used to designate the corresponding status.

[SOURCE: ISO 9000:2015, 3.8.13, modified — Note 1 and Note 3 where not taken over.]

3.21
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.

[SOURCE: ISO 9000:2015, 3.8.12, modified — Note 1 and Note 2 where not taken over.]

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;

— reviewing documents prior to issue.

3.22
warm ischemia
condition before the tissue is removed from the body, but where it is deprived of its normal blood supply

3.23
workflow
series of activities necessary to complete a task

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 examination test performance shall be performed and mitigation measures shall be established to enable the required examination test performance.

The stability of the specific proteins to be examined and their posttranslational 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 [8]).

Before tissues are stabilized by freezing, 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 freezing. In addition, the described effects can vary in different donors'/patients' tissues.

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

NOTE Prolonged cold ischemia durations result in changes of protein (e.g. cytokeratin 18) and phosphoprotein (e.g. phospho-p42/44) amounts[8][9]. Keeping the specimen on wet-ice diminishes this effect[10]. Protein amounts as well as posttranslational modifications can also vary during the pre-examination phase, depending on the origin and type of tissue, the underlying disease, the surgical procedure, the drug regimen, 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 temperatures of the specimen container's surroundings shall be documented. Where the specimen is transported to another facility for freezing, the transport duration shall be documented and the ambient conditions should also be documented.

Safety regulations on transport and handling shall be followed (see 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 manufacturer's instructions, responsibility for its use and performance lies with the user.

5 Outside the laboratory

5.1 Specimen collection

5.1.1 General

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

See also ISO 15189:2012, 5.4.4.

5.1.2 Information about the specimen donor/patient

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

The documentation should include, but is not limited to:

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

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

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

5.1.3 Information about the specimen

The documentation shall include, but is not limited to:

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

NOTE Not needed where small tissue biopsy resection for freezing is performed.

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;

If the freezing of the tissue is performed outside the laboratory, the documentation of steps described under 6.2, where pathology evaluation is required, and 6.3 has to be performed.

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

5.1.4 Specimen processing

Tissues that need to be frozen for diagnostic purposes can originate from a large tissue specimen or can be a small tissue specimen like biopsies e.g. taken by endoscopy or taken from patients during a surgical procedure where fast frozen section diagnosis is required.

NOTE Post-mortem tissues can be frozen for diagnostic purposes. However, preservation of protein is dependent on the time interval between death and autopsy and the temperature of storage of the body after death.

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)]) shall be documented.

Where a pathology diagnosis is required on the specimen, sampling shall be performed by or under supervision or guidance of a medically qualified (e.g. board certified) pathologist (see 6.2).

Where the specimen was removed without the requirement of a pathology diagnosis, the evaluation, selection and documentation of specimens may be done by other qualified persons than pathologists.

The freezing of the specimen or samples taken from the specimen can be performed outside the laboratory or inside the laboratory.

Cold ischemia can influence the protein profile; therefore direct freezing should be preferred.

a) In case the specimen or sample is frozen outside the laboratory, proceed with 6.2 without delay.

b) In case the specimen or sample is frozen inside the laboratory, fresh tissue specimens need to be transported to the laboratory. The steps described under 5.2 for fresh tissue transport shall be performed without delay.

5.2 Fresh tissue transport requirements

5.2.1 General

Where transport of the specimen or sample to the laboratory is required for freezing, the laboratory in collaboration with the clinical or surgery department shall establish a protocol for the transport procedure of the tissue.

5.2.2 Preparations for the transport

The following steps shall be performed:

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

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

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

c) the labelling of the container (e.g. registration-number, barcode, specimen type, quantity, and organ tissue of origin) and additional documentation [information as specified in 5.1.2, 5.1.3, 5.1.4 and 5.2.2 a) and b)].

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

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

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

5.2.3 During transport

Temperature monitoring should be applied in a suitable manner.

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

NOTE There is evidence that proteins in tissues can be stabilized in plastic bags under vacuum and when kept at 0 °C to 4 C° during transport before the samples are frozen for biobanks or used for histopathological evaluation[11].

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

6 Inside the laboratory

6.1 Information about the reception of the specimen

The 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, vacuum integrity) of the received specimens shall be documented. Any deviations from the established protocol for the transport procedure (see 5.2) shall be documented.

The correct identity of the specimen shall be checked. This should include the clinical information (see 5.1.2 and 5.1.3) of the specimen, hospital admission number and/or donor/patient ID, name of the patient, date of birth of the patient.

6.2 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 optional further research purposes shall be done by or under supervision of a medically qualified (e.g. board certified) pathologist to ensure that the collection of the sample for protein examination does not compromise the histopathological analyses. 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.

   NOTE 1 Depending on local procedures, the selection of appropriate parts of the specimen can also be done outside of the laboratory, e.g. in the operating theatre (see 5.1.4).

   In the context of the macroscopic evaluation of the surgical specimen before and/or after freezing, 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 and in correlation with the clinical information and questions. The anatomic localization represented in the specimen shall be described, resection margins and other important areas may be marked if necessary and helpful for later microscopic evaluation; photographs may be taken. Representative samples for microscopic evaluation shall be taken (i.e. grossing) according to the organ/disease specific guidelines from the respective medical societies.

   NOTE 2 The above described evaluation or documentation can also be done outside of the laboratory, e.g. in the operating theatre.

b) When the specimen was removed without the requirement of histopathological diagnosis; the evaluation, selection and documentation of specimens may be done by other qualified persons than pathologists.

c) When frozen section diagnosis is required; the selected part of the specimen shall be frozen (see 6.3) in an appropriate freezing medium. The freezing medium used shall be documented. Frozen sections shall be evaluated by a medically qualified (e.g. board certified) pathologist. Microdissection of tissue should be considered to select or enrich for certain cellular features of a disease.

6.3 Freezing of the specimen or sample(s)

6.3.1 Freezing of the specimen or samples shall be performed without delay. The most optimal freezing method (see 6.3.2) for the specimen or sample in regards to the intended use and the working conditions should be chosen and used.

The vial shall be labelled before it is pre-cooled.

6.3.2 The three possible freezing procedures are as follows:

a) Snap-freezing procedure[12]: This method is used in frozen tissue samples. Isopentane (C_5H_{12}, also called methylbutane or 2-methylbutane) shall be pre-cooled ranging from ≤−80 °C to >−160 °C where it can be used for snap freezing. Pre-cooling can be done with liquid nitrogen (−196 °C), dry ice (−80 °C), −80 °C freezers or dedicated freezing appliances that keep the isopentane ≤−80 °C with or without controlled cooling rate. The isopentane shall be cooled in a tube or other container (e.g. glass beaker) resistant to the large and sudden temperature shifts. The volume of pre-cooled isopentane shall be at least 10 times the volume of the specimen or sample. For snap freezing the tissue sample shall be completely submerged into the pre-cooled isopentane.

   After the tissue is frozen it shall be transferred into its designated pre-cooled labelled cryo-vial. The vial shall be closed according to the manufacturer's instructions. The isopentane should be refreshed when tissue sediment is seen at the bottom of the tube.

Isopentane is an extremely volatile and extremely flammable liquid at room temperature and pressure. Therefore, the laboratory should be well ventilated. The isopentane in the tube should be cooled.

b) Fast freezing procedure: Tissues shall be fast frozen on a precooled metal plate, or metal basket placed on the surface of liquid nitrogen, or on dry ice. The metal surface shall be precooled ranging from ≤−80 °C to >−196 °C. The metal plate or metal basket can be fixed into position with a suitable stand and clamp. Alternatively the sample can be frozen directly in liquid nitrogen or even in the labelled and closed storage vial in liquid nitrogen or in dry ice. However, a slow freezing process can cause membrane disruption by compartmental rising salt concentrations and crystal formation which can seriously affect the morphology. To avoid cross-contamination, the basket or plate should be cleaned between freezing samples.

NOTE Freezing in liquid nitrogen is characterized by the Leidenfrost effect[13] caused by boiling of liquid nitrogen around the tissue due to its relatively high temperature. This reduces the heat conduct from the sample to the liquid nitrogen; this becomes worse when the sample is placed in the labelled vial.

c) Frozen section procedure: For freezing tissue for fast frozen section diagnosis, the tissue should be transported freshly to the laboratory without delay. The selected part of the specimen shall be frozen onto the specialized metal grids that fit onto the cryostat in an appropriate freezing medium. The freezing medium used should be documented. The metal grid containing the tissue and freezing medium is frozen by holding the metal in liquid nitrogen or dry ice until the tissue is frozen. After cutting the frozen sections the remainder should be removed from the metal grid without thawing and stored in a pre-cooled vial for long term storage.

NOTE The use of a freezing medium is known to harm protein mass spectrometry where the column separation can become impaired.

6.3.3 The following steps shall be performed before, during and after the freezing procedure:

a) the documentation of the freezing procedure (e.g. freezing in liquid nitrogen, snap-freezing in isopentane cooled by liquid nitrogen or dry ice, freezing in an appropriate freezing medium, freezing with controlled cooling rate);

b) the documentation of the freezing time point and date (to determine the lag time: time period between removal from the body – until freezing of the specimen or sample);

c) determine if the required tissue size of the sample fits into the chosen cryo-vial before freezing, because the tissue size determines the size of the container; it is therefore recommended, that the specimen/sample does not exceed 1 cm in one dimension;

d) the selection of the specimen/sample container for cryo-storage:

   1) the container shall have a sufficient volume for the size of the specimen or sample to be stored;

   2) the container shall be certified for the storage temperature;

   3) the container shall be safely closable, preferably screw caps; containers with a flip cap shall not be used;

      NOTE Containers can explode upon specimen or sample retrieval when they have been stored in liquid nitrogen.

   4) the container shall be suited for permanent labelling under the frozen storage conditions;

e) the label shall be suitable for the respective frozen storage conditions;

NOTE Suitable labels are e.g. self-adhesive labels, handwriting, radio frequency identification (RFID), pre-labelled containers , which have been verified for the purpose.

f) the labelling of the container shall ensure appropriate traceability of specimens and samples. Therefore, the container labelling shall provide the minimum information of:

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

   2) the basic information on e.g. the tissue type, tissue and disease condition 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 6.3.3 f) 1);

   3) the unique numbering of each container, which can be included in 6.3.3 f) 1);

g) the documentation of types, quantity and description of the specimen(s) or sample(s).

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

6.4 Storage requirements

The constant temperature shall be ≤–70 °C. Systems monitoring and controlling the temperature should be implemented and used.

Freezers or liquid nitrogen tanks shall have a temperature alarm system.

Major temperature shifts may occur during retrieval of the specimen(s) or sample(s). Therefore, retrieval times should be kept as short as possible to avoid the thawing of samples.

Temperature shifts occurring that accidentally may have thawed the specimen(s) or sample(s) to be processed or to be further stored, shall be documented.

Back-up cryo-storage facilities should be provided.

The storage position, storage temperature, time and date of the retrieval of any specimen or sample from the storage system shall be documented.

6.5 Isolation of total protein

6.5.1 General

A histopathological characterization of the cellular composition and disease condition of the specimen or sample shall be performed (e.g. on hematoxylin sections) according to an internationally defined histopathological classification (e.g. WHO/IARC Classification of Tumours[14]). 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.

The specimen or sample shall not thaw before its homogenous dispersion in lysis buffers containing suitable substances inhibiting degradation or modification of proteins, such as inhibitors for proteases, kinases, or phosphatases. The specimen and/or sample shall be thoroughly minced in its frozen state and thoroughly dispersed with lysis buffers containing the previously mentioned inhibiting substances. The homogenization of the frozen specimen or sample in the lysis buffer shall therefore be processed immediately after having introduced the tissue into the lysis buffer.

In case, that the processed specimen or sample contains freezing medium, this shall be documented.

All tools, such as forceps, used to manipulate the frozen sample for homogenization or cryo-sectioning or transferring into the lysis buffer shall be clean to minimize contamination with protease, kinase, or phosphatases and should be cooled to at least –20 °C before use. Histotechnologists shall wear gloves. The relevant parts of the microtome, including the reusable blade, shall be cleaned after the cutting of each frozen tissue specimen/sample. The use of new disposable blades on the microtome should be considered to avoid cross-contaminations.

If there is doubt in the correct identification of the specimen or sample, an identification verification test shall be performed.

The isolation of protein is a key step in the diagnostic workflow, which shall be especially focused on during the validation of the entire workflow.

6.5.2 Using commercial kits

Where using commercial kits dedicated to the isolation of protein from frozen tissues, the manufacturer's instructions for use shall be followed.

6.5.3 Using the laboratories own protocols

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

6.5.3.2 If the laboratory uses its own protocol independently from a commercial kit, the validation demonstrating that it is fit for purpose shall be done and instructions shall be written and followed.

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

6.6 Quantity and quality assessment of isolated proteins

The protein quality and quantity should be checked according to the diagnostic kit manufacturer's instructions, or where provider's instructions are not available, by generally accepted physical, chemical or biochemical procedures (e.g. Western blot[15], Bradford assay[16]), and/or by suitable controls as part of the examination.

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

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. beta-actin).

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

a) Bradford assay;

b) bicinchoninic acid (BCA) assay;

c) Lowry assay.

6.7 Storage of isolated total protein

The protein isolation kit provider's specific instructions for storing the isolated protein should be followed. Where the examination provider’s instructions are more stringent, these 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.

For long-term storage, aliquots of the isolated protein should be generated to avoid repeated freezing and thawing. The aliquots should not be further diluted to avoid a reduction of the protein stability. Avoid more than two freeze–thaw cycles, use aliquots instead. If lyophilized, proteins can be stored for several years at 4 °C or –20 °C.

NOTE 2 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 protein 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)

Quantitative protein examination 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 are being used to develop targeted therapies directed against deregulated signalling pathways in cancer patients. However, knowledge of the impact of pre-examination variations, such as delayed time to tissue freezing, on protein and phosphoprotein changes 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 frozen tissues and the subsequent isolation and storage of proteins and phosphoproteins before the quantitative analysis. 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 duration 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 may be unreliable and meaningful biomarkers for treatment of patients may be missed or interpreted wrongly.

A.2 Example

A.2.1 General

Human intestine and liver tissues were used in a time course experiment to assess the influence of prolonged cold ischemia on the amounts of proteins and phosphoproteins in the specimens before freezing. The data revealed that the protein and phosphoprotein amounts changed before the tissues are stabilized by freezing.

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 the 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 the post-resection samples from most patients'. In contrast, amounts of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin were found to be stable during prolonged cold ischemia times.

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 freezing, typically the transport time (2) to the pathology laboratory, is

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