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BSI Standards Publication

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

Part 2: Isolated proteins

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Molekularanalytische in-vitro-diagnostische Verfahren - Spezifikationen für präanalytische Prozesse für FFPE-Gewebeproben - Teil 2: Isolierte Proteine

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European foreword

This document (CEN/TS 16827-2:2015) has been prepared by Technical Committee CEN/TC 140 "*In vitro* diagnostic medical devices", the secretariat of which is held by DIN.

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Introduction

Molecular in vitro diagnostics has enabled a significant progress in medicine. Further progress is expected by new technologies analysing signatures of nucleic acids, proteins, and metabolites in human tissues and body fluids. However, the profiles and/or integrity of these molecules can change drastically during primary sample collection, transport, storage, and processing thus making the outcome from diagnostics or research unreliable or even impossible because the subsequent analytical 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 extraction techniques from formalin 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 extraction procedures [1], [2]. Currently, most investigators accept that proteins extracted from FFPE tissue are suitable for downstream proteomic analysis [3].

However, a standardization of the entire process from primary sample collection to protein analysis is needed. Studies have been undertaken to determine the important influencing factors. This Technical Specification draws upon such work to codify and standardise the steps for FFPE tissue with regard to protein analysis in what is referred to as the preanalytical phase.

1 Scope

This Technical Specification gives recommendations for the handling, documentation and processing of FFPE tissue specimens intended for the analysis of extracted proteins during the preanalytical phase before a molecular assay is performed. This Technical Specification is applicable to molecular *in vitro* diagnostic examinations (e.g., *in vitro* diagnostic laboratories, laboratory customers, developers and manufacturers of *in vitro* diagnostics, institutions and commercial organizations performing biomedical research, biobanks, and regulatory authorities).

Protein profiles and protein-protein interactions in tissues can change drastically before and after collection (due to e.g., gene induction, gene down regulation, protein degradation). Protein species amounts can change differently in tissues from different donors / patients. 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 environment conditions after the tissue removal from the body.

Furthermore, the formalin fixation and paraffin embedding process leads to modifications of the protein molecules, which can impact the validity and reliability of the analytical test results.

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

This document is not applicable for protein analysis by immunohistochemistry.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 15189:2012, *Medical laboratories — Requirements for quality and competence (ISO 15189:2012, Corrected version 2014-08-15)*

ISO 15190, *Medical laboratories — Requirements for safety*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in EN ISO 15189:2012 and the following apply.

3.1
ambient temperature
unregulated temperature of the surrounding air

3.2
analytical phase
processes that start with the isolated analyte and include all kinds of parameter testing or chemical manipulation for quantitative or qualitative analysis

3.3
cold ischemia
condition after removal of the tissue from the body until its stabilization or fixation

3.4
FFPE
formalin fixation and paraffin embedding

3.5
FFPE tissues
formalin fixed and paraffin embedded tissues

3.6
formalin
saturated formaldehyde solution containing a mas fraction of 37 % (corresponding to a volume fraction of 40 %) formaldehyde, termed 100 % formalin

3.7
formalin fixation
treatment of a sample with standard buffered formalin solution for stabilization

3.8
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, surgical procedure, collection of the primary sample(s), temporary storage, transportation to and within the analytical laboratory, aliquoting, retrieval, isolation of analytes, and end when the analytical examination begins

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

Note 1 to entry: The preanalytical phase may include preparative processes that may influence the outcome of the intended examination.

3.9
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: EN ISO 15189:2012, 3.16, modified — The term and definition is used here without the original notes.]

3.10
protein
type of biological macromolecules composed of one or more chains with a defined sequence of amino acids connected through peptide bonds

3.11
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

3.12
protein species
amounts of a chemically clearly-defined protein corresponding to one spot on a high-performance 2-dimensional gel electrophoresis pattern

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

3.13
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]

3.14
room temperature
temperature which is defined as 18 °C to 25 °C for the purposes of this document

3.15
sample
one or more parts taken from a primary sample

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

3.16
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 30:1992, 2.7]

Note 1 to entry: The measured constituent for the purpose of this document is RNA.

3.17
standard buffered formalin solution
10 % formalin solution containing 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 methanol to inhibit oxidation and polymerization of formaldehyde.

3.18
warm ischemia
warm Ischemia is the condition where the tissue is deprived of its normal blood supply containing oxygen and nutrients while the tissue is at body temperature

4 General considerations

For general statements on primary sample collection and handling (including avoidance of cross contaminations) see EN ISO 15189:2012, 5.4.4, 5.2.6. Consumables including kits shall be verified before use in examination (see EN ISO 15189:2012, 5.3.2.3); EN ISO 15189:2012, 5.5.1.2 and 5.5.1.3 can also apply.

As all steps of a diagnostic workflow can influence the final analytical performance, the entire workflow comprising the preanalytical steps, including information on biomolecule stability and storage conditions, and analytical steps should be verified and validated (see EN ISO 15189).

The stability of the specific protein(s) of interest and their posttranslational modifications (if important for the assay) should be investigated throughout the complete preanalytical workflow prior to the development and implementation of an analytical test.

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 tissues from different donors / patients.

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

NOTE Prolonged cold ischemia times result in changes of protein (e.g., cytokeratin 18) and phosphoprotein (e.g., phospho-p42/44) amounts [4], [5]. Keeping the specimen on wet-ice diminishes this effect [6]. Proteins amounts as well as the protein modifications can 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 time and duration should be documented. When it is not possible to avoid cold ischemia, its time of onset and duration shall be documented and the temperatures of the specimen transport container's surroundings should 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, formalin fixation causes modifications of biomolecules and leads to suboptimal performance of protein extracted from FFPE tissues [7] that should be considered in quality control and application of molecular assays. Assay optimization for FFPE tissues or the use of non-crosslinking alternatives to standard buffered formalin solution is an option to minimize this issue for molecular analyses.

Safety regulations on transport and handling shall be considered (see EN ISO 15189:2012, 5.2.3 and 5.4.5 and ISO 15190).

During the whole preanalytical workflow precautions shall be taken to avoid cross contamination between different 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 Primary tissue collection manual

5.1.1 Information about the primary sample donor

The documentation should include, but is not limited to:

a) the primary donor / patient ID, which can be in the form of a code;

b) the health status of the primary sample donor (e.g., healthy, disease type, concomitant disease);

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

d) the start of ischemia within the body (warm ischemia) by documenting the ischemia-relevant vessel ligation/clamping time point (usually arterial clamping time).

5.1.2 Information on the primary tissue sample

The documentation shall include, but is not limited to:

a) the time point when tissue is removed from the body;

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

c) the documentation steps described under 6.2, if the formalin fixation starts outside the laboratory.

5.1.3 Information on the primary tissue sample processing

The following steps shall be performed:

1. the documentation of any additions or modifications to the primary sample after removal from the body (e.g., labelling for the orientation of the specimen (e.g., ink-marking, stitches), incision(s));

2. the selection and use of transport containers and packages (e.g., cooling box, box for storing and transportation, vacuum packaging) fit for transport of formalin fixed tissue samples, if relevant;

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

   NOTE 1 Accidentally freezing and thawing 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).

4. the labelling of the transport container (e.g., registration-number, barcode (1D or 2D), primary sample type, quantity, and organ tissue of origin) and additional documentation (information as specified in 5.1.1, 5.1.2, and 5.1.3, 1. to 3.). If a single sample container contains several aliquots of the same specimen, and the aliquots represent different features (e.g., tissue type, disease status, location) this shall be documented.

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

The temperatures of the transport container´s surroundings during cold ischemia time (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 partnership with the clinical or surgery department shall establish a protocol for the transport procedure of the specimen.

If the primary tissue sample 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 stabilised in plastic bags under vacuum when kept at 0 °C to 4 °C during transport [8] before the samples are archived for biobanks or used for histopathological evaluation.

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

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

6 Inside the laboratory

6.1 Information on the primary tissue sample receipt

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

6.2 Formalin fixation of the specimen

The fixative used should be standard buffered formalin solution.

The pH-value and concentration of the standard buffered formalin solution should be checked regularly as formalin is not stable (e.g., it has a tendency to be oxidized to formic acid [6]).

The following steps shall be performed:

1. the consultation with the manufacturer's material safety data sheet (MSDS) before handling standard buffered formalin solution;

   NOTE Formaldehyde is a hazardous compound that chemically modifies biomolecules.

2. the documentation of the time point of placing the tissue sample into standard buffered formalin solution;

   NOTE The total formalin fixation duration can impact further analyses, e.g., immunohistochemical techniques, nucleic acid based molecular analyses. The optimal formalin fixation duration can vary depending on tissue type and size. For larger surgical specimen inhomogeneous fixation can occur before the grossing process due to the slow penetration rate 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 analytical test. It has been shown that the protein yield decreased with increasing fixation times [10], [11].

   EXAMPLE For tissue pieces with a maximum thickness of 5 mm fixation times between 12 h and 24 h are reasonable in most cases. See also 6.7.2.

3. the selection of collection container/s:

   a) the capacity of the collection containers should be such that the primary sample 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 4:1, (volume to volume). To ensure complete formalin fixation of larger specimens a special tissue handling such as incision(s) of solid organs or opening of hollow organs can be necessary.

   NOTE The fixative-to-tissue-volume-ratio influences the progression of the fixation. There is evidence that a ratio of 10:1 can be necessary [9]. If the container has a small volume, standard buffered formalin solution can be changed periodically, because formalin reacts with tissue components in a time-dependent way.

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

   c) the container shall be securely closable;

4. the labelling of the container (e.g., by using self-adhesive labels, handwriting, radio frequency identification devices (RFID), pre-labelled containers, bar codes) shall give the minimum information of:

   a) the patient ID, which can be in the form of a code;

   b) the basic information on 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 sample used in 6.2, 4. a);

   c) the unique numbering of each container;

5. the documentation of types, quantity and description of samples.

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 analysis is evaluated by a medically qualified (e.g., board certified) pathologist. In this context it should be documented which features of a disease are actually reflected in the tissue sample used for molecular analysis (e.g., different molecular mechanisms can be activated at 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

The evaluation and documentation of the pathology of the specimen and the selection of the sample 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 may apply.

Options to select the sample for protein analysis:

a) The selection of appropriate parts of the specimen for molecular analyses and histopathological analyses 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 analysis does not compromise the histopathological analyses.

   In the context of macroscopic evaluation of the surgical specimen before and/or after formalin fixation the clinical instructions, number, 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 instructions and questions. The anatomic localization represented in the specimen shall be described, resection margins and other important areas may be marked if necessary and be helpful for later microscopic evaluation; photographs may be taken. Where representative samples for microscopic evaluation are required (i.e., grossing) this shall be done in accordance with the organ- / disease-specific guidelines from the respective medical societies.

b) Where the tissue specimen was removed from the body without the requirement of histopathological diagnosis; documentation of this specimen, the evaluation, as well as the documentation and selection of the samples may be done by other qualified persons than pathologists.

   NOTE Documentation can include photographs.

The size of the samples should 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 aliquots of the same specimen, and the aliquots 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) as part of the embedding process.

The total duration of formalin fixation and the temperature during the fixation process shall be documented.

6.4 Post-fixation of frozen samples

Frozen samples (e.g., after frozen section diagnosis) can be post-fixed in standard buffered formalin solution for paraffin embedding.

The total formalin fixation duration shall be documented.

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

6.5 Processing and paraffin embedding

Where the sample had been placed into standard buffered formalin solution, the time point when it is placed into an alcohol-containing solution of the embedding process shall be documented.

Further processing shall be performed in a tissue processor / embedding machine according to the manufacturer´s instructions.

NOTE During processing, the tissue sample is dehydrated and water is replaced with paraffin wax. It is essential to replace water quantitatively, since residual water leads to tissue degradation during storage [12].

The replacement of all reagents in the tissue processor / embedding machine shall be done on a regular basis according to the manufacturers' instructions.

The duration and temperature of paraffin infiltration impact the biomolecule stabilization in fixed tissue. Low melting paraffin for infiltration should be used. The duration within the melted paraffin should be restricted to the time required by the validated protocol. Type of paraffin, incubation temperature and duration should be documented.

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 analytical test shall therefore be validated for the paraffin embedding processes used. If impacts on the analytical test are recognized, the temperature and/or the duration should be reduced.

6.6 Storage requirements

FFPE tissue samples can be stored in several ways, e.g., as blocks, cut sections or as multi tissue arrays [13]. Storage time may influence the retrieval of a protein or group of proteins over time and thus impact the subsequent proteomic measurements [14]. While histology is hardly affected by storage, protein yield may decrease with increasing storage time, especially if samples are stored for years [10].

Storage conditions, e.g., humidity and temperature, can have an impact on protein amounts in archival FFPE tissues [13].

NOTE 1 One study reported a decreased Western blot signal of proteins extracted from FFPE tissue sections depending on the storage temperature and humidity [15].

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

NOTE 2 Lower storage temperatures (e.g., +4 °C, -20 °C) slow down the protein degradation process over time.

NOTE 3 If the FFPE blocks are not stored dry, the protein degradation can increase and fungi can grow.

For protein extractions 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 2 °C to 8 °C or lower temperature for as short as possible.

A system for archiving paraffin blocks should be in place. The storage position, e.g., drawer number, row number, place, and the time of the retrieval from the storage system shall be documented.

6.7 Isolation of the total protein

6.7.1 General

Where a histopathological characterization of the cellular composition and disease condition of the sample was not performed under 6.3, and is needed, it shall be performed at this stage to assess the cellular composition and disease condition.

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 analytical test.

Requirements and recommendations:

1. The optimal fixation duration depends on the sample type and size. In general, it should be 12 h to 24 h in standard buffered formalin solution for a sample thickness of up to 5 mm [13]. Prolonged tissue fixation times, which can result in reduced protein yields, should be avoided.

2. Starting material for protein purification should be freshly cut sections, with a thickness of up to 10 µm, obtained from trimmed FFPE tissue blocks.

3. Parallel haematoxylin/eosin (H&E) stained sections should be used to identify, select and control dissection of unstained specimens for subsequent protein extraction and quantification. Staining sections prior to protein extraction should not be performed, as staining can impair protein quality and performance in downstream applications [16].

The laboratory shall validate the process from the isolation of protein to the final analytical test result according to its internal quality management system (see EN ISO 15189).

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

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

6.7.4.2 If the laboratory uses its own protocol independent from a commercial kit, the validation demonstrating fit for purpose shall be done, and instructions shall be written and followed.

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

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,

Protein isolation procedures from FFPE tissue sections mounted directly onto a glass slide should contain the following three steps:

1. 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.

2. 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 Suitable paraffin wax solvents are e.g., xylene (where not prohibited), heptane, or limonene.

3. Protein extraction.

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

NOTE 1 Depending on the subsequent analytical test (e.g., mass spectrometry) additional steps (e.g., generation of peptides) may be required after the removal of the detergent.

NOTE 2 Partial solubilisation of FFPE tissues can lead to extraction bias and can affect the final assay result [3].

6.8 Quantity and quality assessment of isolated RNA

The protein quality and quantity shall be checked up-front the analytical test by generally accepted physical, chemical or biochemical procedures (e.g., Western blot [17], Bradford assay [18]), and/or by suitable controls being part of the analytical test.

6.9 Storage of isolated RNA

The laboratory shall have verified procedures in place for storing isolated total protein. Where there are specific instructions supplied by a protein isolation kit provider for storing and archiving isolated protein, these shall be followed.

If there is no information available from the protein isolation kit provider or if 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 (e.g., 2 h) can be feasible in certain circumstances.

Storage for archiving purposes (i.e., for several years) should be at -20 °C, -70 °C or below. 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.

Appropriate storage vessels, such as cryovials, should be used.

Annex A
(informative)

Quality control of RNA extracted from formalin fixed and paraffin embedded tissue samples: implications for RT-qPCR based analyses[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 preanalytical variations, such as delayed time until 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 preanalytical 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 extraction and storage of proteins and phosphoproteins before the quantitative analysis. This standardization process includes the documentation of warm and cold ischemia times. While the results for warm ischemia are reported elsewhere (see further reading), the data shown here indicate that cold ischemia time has an influence on protein profiles. Thus, there is a risk that due to variations in warm and cold ischemia times and other preanalytical parameters the analytical 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 were 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 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 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 times.

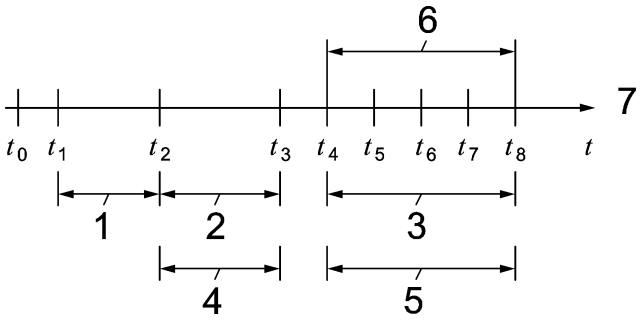
A.2.2 Experimental procedures

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 formalin fixation, typically the transport time (2) to the pathology laboratory, is defined

1) Research by the EU FP/ SPIDIA project funded by the European Union Seventh Framework Programme [FP7/2007-2013] under grant agreement no 222916.

as cold ischemia (4). A time course experiment with experimental delay to formalin fixation (3) and t_3 as reference sample was performed on aliquoted samples stored in a humidified chamber at room temperature in which at different intervals (t_4 to t_8) samples were formalin fixed and paraffin embedded, resulting in experimentally delayed cold ischemia times (5).



Key

1	warm ischemia	t_1	vessel ligation
2	transport	t_2	surgical resection
3	experimental delay to formalin fixation	t_3	formalin fixation of the reference sample
4	cold ischemia	t_4	formalin fixation of aliquot sample 1
5	experimentally delayed cold ischemia	t_5	formalin fixation of aliquot sample 2
6	different time points in the laboratory until formalin fixation	t_6	formalin fixation of aliquot sample 3
7	molecular analysis	t_7	formalin fixation of aliquot sample 4
t	time	t_8	formalin fixation of aliquot sample 5
t_0	start of surgery		

Figure A.1 — Overview of tissue collection

A.2.2.2 Tissues

To study the impact of cold ischemia time on protein and phosphoprotein amounts, non-malignant human intestine and liver samples were collected during routine surgical procedures. Specimens were transported at room temperature to the Institute of Pathology for further processing. Each specimen was aliquoted into samples of minimum size of 5 mm × 5 mm × 5 mm and placed into standard buffered formalin solution. The reference sample was fixed immediately after arrival at the pathology laboratory. All other samples were kept in a humidified chamber at room temperature for 30 min to 360 min, simulating an experimental delay to formalin fixation and paraffin embedding (Figure A.1).

A.2.2.3 Protein analysis

Reverse phase protein array (RPPA) and Western blot analysis were applied as target-specific approaches to analyze single proteins and amounts of certain phosphorylated proteins. The antibodies used are listed in Table A.1.

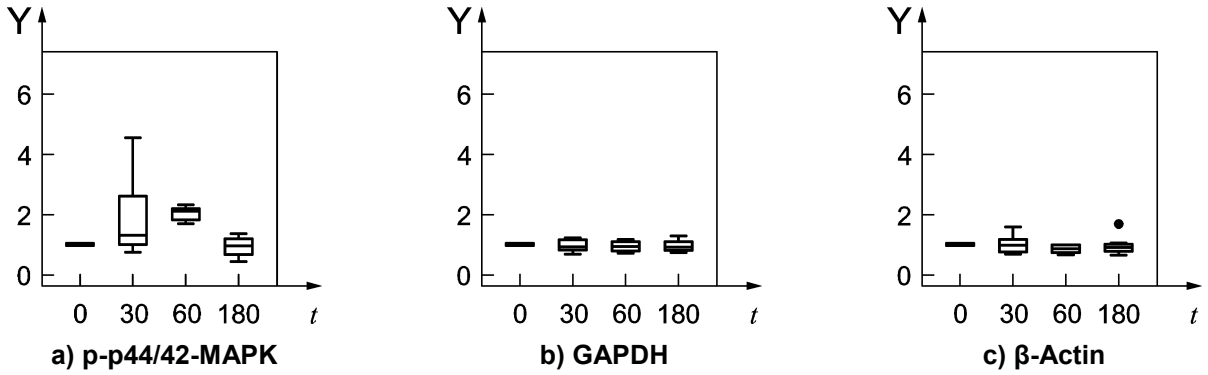
Table A.1 — Antibodies used for RPPA and Western blot analysis

Protein (n=23)	Species	Dilution
Phospho-PTEN (Ser380)	rb	1:500 / 5 % BSA / TBST
PTEN	rb	1:500 / 5 % BSA / TBST
Phospho-Akt (Ser473)	rb	1:500 / 5 % BSA / TBST
Akt	rb	1:1 000 / 5 % BSA / TBST
Phospho-GSK-3beta (Ser9)	rb	1:500 / 5 % BSA / TBST
GSK-3beta	rb	1:500 / 5 % BSA / TBST
Phospho-p44/42 MAPK (Thr202/Tyr204)	rb	1:500 / 5 % BSA / TBST
p44/42 MAPK	rb	1:1 000 / 5 % BSA / TBST
Phospho-p38 MAPK (Thr180/Tyr182)	rb	1:500 / 5 % BSA / TBST
p38 MAPK	rb	1:500 / 5 % BSA / TBST
Phospho-Stat3 (Ser727)	rb	1:1 000 / 5 % BSA / TBST
Stat3	rb	1:1 000 / 5 % BSA / TBST
Phospho-NF-kappaB p65 (Ser536)	rb	1:500 / TBST
NF-kappaB p65	rb	1:1 000 / 5 % BSA / TBST
FAK	rb	1:1 000 / 5 % BSA / TBST
c-Fos	rb	1:500 / 5 % BSA / TBST
Cleaved Caspase-3	rb	1:500 / 5 % BSA / TBST
E-Cadherin	ms	1:1 000 / 5 % BSA / TBST
Egr-1	rb	1:1 000 / 5 % BSA / TBST
HIF-1 alpha	rb	1:500 / 5 % BSA / TBST
Hsp70	rb	1:500 / 5 % BSA / TBST
β-Actin	ms	1:1 000 / 5 % BSA / TBST
GAPDH	ms	1:1 000 / 5 % BSA / TBST
NOTE The explanations for the used abbreviations are: rb (rabbit), ms (mouse), M (milk), BSA (bovine serum albumin), TBST (Tris-buffered saline with 0,1 % Tween-20).		

A.2.3 Results

To investigate the impact of cold ischemia time on protein profiles, human non-malignant tissue specimens (n = 11) were collected during routine operating procedures and transported to the pathology laboratory where controlled time course experiments were initiated upon arrival. After protein extraction, the stability of selected proteins was quantitatively analysed by reverse phase protein array (RPPA) with 23 specific antibodies, seven of which were directed against phosphorylated epitopes, and one recognizes a cleaved protein. The proteins were selected based on their potential involvement in critical cellular functions or signalling pathways, such as cell structure, proliferation and survival, stress response, hypoxia, apoptosis, and adhesion. We thought by analysing protein cleavage and post-translational modifications (phosphorylation) we can reliably determine potential fluctuations in protein and phosphoprotein amounts during ischemia times.

Figure A.2 shows the signal intensities of three representative proteins (phospho-p44/42-MAPK, GAPDH, β-actin). Depicted are box plots which show the median normalized signal intensity and the percentiles, relative to the reference sample (see Figure A.1, t_3) which was set at 1,0.



Key

Y relative mean intensity X time (min)

Figure A.2 — Analysis of proteins and phosphoproteins during experimentally delayed cold ischemia

Phospho-p44/42-MAPK (p-p44/42-MAPK) is an example of a phosphoprotein which amounts change during cold ischemia, while the amounts of GAPDH and β-actin do not seem to change during the time course experiment.

A.2.4 Further reading

Gündisch S., Hauck S., Sarioglu H., Schott C., Viertler C., Kap M., Schuster T., Reischauer B., Rosenberg R., Verhoef C., Mischinger H. J., Riegman P., Zatloukal K., Becker K.-F. Variability of protein and phosphoprotein levels in clinical tissue specimens during the preanalytical phase. Journal of Proteome Research. 2012 Dec 7; 11(12):5748-62.

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Bibliography

[1] Ikeda K., Monden T., Kanoh T., Tsujie M., Izawa H., Haba A., Ohnishi T., Sekimoto M, Tomita N, Shiozaki H, Monden M. et al. Extraction and analysis of diagnostically useful proteins from formalin-fixed, paraffin-embedded tissue sections. The journal of histochemistry and cytochemistry: official journal of the Histochemistry Society. 1998; 46: pp. 397-403.

[2] BECKER K.F., SCHOTT C., HIPP S., METZGER V., PORSCHEWSKI P., BECK R. et al. Quantitative protein analysis from formalin-fixed tissues: implications for translational clinical research and nanoscale molecular diagnosis. *J. Pathol.* 2007, **211** (3) pp. 370–378

[3] SHI S.-R., TAYLOR C.R., FOWLER C.B., MASON J.T. Complete solubilization of formalin-fixed, paraffin-embedded tissue may improve proteomic studies. *Proteomics Clin. Appl.* 2013, **7** (3-4) pp. 264–272. DOI:10.1002/prca.201200031

[4] ESPINA V., EDMISTON K.H., HEIBY M., PIEROBON M., SCIRO M., MERRITT B. et al. A portrait of tissue phosphoprotein stability in the clinical tissue procurement process. *Mol. Cell. Proteomics*. 2008 Oct, **7** (10) pp. 1998–2018. DOI:10.1074/mcp.M700596-MCP200

[5] GÜNDISCH S., HAUCK S., SARIOGLU H., SCHOTT C., VIERTLER C., KAP M. et al. Variability of protein and phosphoprotein amounts in clinical tissue specimens during the preanalytical phase. *J. Proteome Res.* 2012 Dec 7, **11** (12) pp. 5748–5762. DOI:10.1021/pr300560y

[6] GÜNDISCH S., GRUNDNER-CULEMANN K., WOLFF C., SCHOTT C., REISCHAUER B., MACHATTI M. et al. Delayed times to tissue fixation result in unpredictable global phosphoproteome changes. *J. Proteome Res.* 2013 Oct 4, **12** (10) pp. 4424–4434. DOI:10.1021/pr400451z

[7] VINCENTI D.C., MURRAY G.I. The proteomics of formalin-fixed wax-embedded tissue. *Clin. Biochem.* 2013 Apr, **46** (6) pp. 546–551. DOI:10.1016/j.clinbiochem.2012.10.002

[8] Condelli V., Lettini G., Patitucci G., D'Auria F., D'Amico M., Vita G. et al. Validation of vacuum-based refrigerated system for biobanking tissue preservation: analysis of cellular morphology, protein stability, and RNA quality. Biopreservation and biobanking. 2014;12(1):35-45.

[9] FOX C.H., JOHNSON F.B., WHITING J., ROLLER P.P. Formaldehyde fixation. *J. Histochem. Cytochem.* 1985, **33** (8) pp. 845–853

[10] WOLFF C., SCHOTT C., PORSCHEWSKI P., REISCHAUER B., BECKER K.-F. Successful protein extraction from over-fixed and long-term stored formalin-fixed tissues. *PloS One Journal.* 2011, **6** (1) p. e16353

[11] TANCA A., PAGNOZZI D., FALCHI G., BIOSA G., ROCCA S., FODDAI G. et al. Impact of fixation time on GeLC-MS/MS proteomic profiling of formalin-fixed, paraffin-embedded tissues. *J. Proteomics*. 2011, **74** pp. 1015–1021

[12] HEWITT S.M., LEWIS F.A., CAO Y., CONRAD R.C., CRONIN M., DANENBERG K.D. et al. Tissue handling and specimen preparation in surgical pathology: issues concerning the recovery of nucleic acids from formalin-fixed, paraffin-embedded tissue. *Arch. Pathol. Lab. Med.* 2008, **132** pp. 1929–1935

[13] THOMPSON S.M., CRAVEN R.A., NIRMALAN N.J., HARNDEN P., SELBY P.J., BANKS R.E. Impact of pre-analytical factors on the proteomic analysis of formalin-fixed paraffin-embedded tissue. *Proteomics Clin. Appl.* 2013, **7** (3-4) pp. 241–251. DOI:10.1002/prca.201200086

[14] BALGLEY B.M., GUO T., ZHAO K., FANG X., TAVASSOLI F.A., LEE C.S. Evaluation of archival time on shotgun proteomics of formalin-fixed and paraffin-embedded tissues. *J. Proteome Res.* 2009, **8** pp. 917–925. DOI:10.1021/pr800503u

[15] Xie R., Chung J.-Y., Ylaya K., Williams R.L., Guerrero N., Nakatsuka N. et al. Factors influencing the degradation of archival formalin-fixed paraffin-embedded tissue sections. The journal of histochemistry and cytochemistry: official journal of the Histochemistry Society. 2011; 59: pp. 356-65.

[16] BECKER K.-F., SCHOTT C., BECKER I., HÖFLER H. Guided protein extraction from formalin-fixed tissues for quantitative multiplex analysis avoids detrimental effects of histological stains. *Proteomics Clin. Appl.* 2008, **2** (5) pp. 737–743. DOI:10.1002/prca.200780106

[17] Gallagher S.R. 2010, Protein Blotting: Immunoblotting, Current Protocols Essential Laboratory Techniques 4:8.3.1-8.3.36. © 2010 by John Wiley & Sons, Inc. DOI: 10.1002/9780470089941.et0803s04

[18] Olson B. J. S. C., Markwell J., Assays for Determination of Protein Concentration. Current Protocols Essential Laboratory Techniques, Unit 3.4, © 2010 by John Wiley & Sons, Inc., DOI: 10.1002/0471140864.ps0304s48

[19] BERG D., HIPP S., MALINOWSKY K., BÖLLNER C., BECKER K.F. Molecular profiling of signalling pathways in formalin-fixed and paraffin-embedded cancer tissues. *Eur. J. Cancer*. 2010 Jan, **46** (1) pp. 47–55. DOI:10.1016/j.ejca.2009.10.016

[20] ISO Guide 30:1992, *Terms and definitions used in connection with reference materials*

[21] ENCYCLOPEDIA OF PSYCHOPHARMACOLOGY. In: *Stolerman*. (IAN P., ed.). Springer, 2010

[22] JUNGBLUT P., THIEDE B., ZIMNY-ARNDT U., MULLER E., SCHELER C., WITTMANN-LIEBOLD B. et al. Resolution power of two-dimensional electrophoresis and identification of proteins from gels. *Electrophoresis*. 1996, **17** pp. 839–847

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