PD CEN/TS 16827-1:2015

BSI Standards Publication

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

Part 1: Isolated RNA

bsi.

...making excellence a habit.™

National foreword

This Published Document is the UK implementation of CEN/TS 16827-1:2015.

The UK participation in its preparation was entrusted to Technical Committee CH/212, IVDs.

A list of organizations represented on this committee can be obtained on request to its secretary.

This publication does not purport to include all the necessary provisions of a contract. Users are responsible for its correct application.

© The British Standards Institution 2015. Published by BSI Standards Limited 2015

ISBN 978 0 580 85029 5

ICS 11.100.10

Compliance with a British Standard cannot confer immunity from legal obligations.

This Published Document was published under the authority of the Standards Policy and Strategy Committee on 31 August 2015.

Amendments issued since publication

Date	Text affected

TECHNICAL SPECIFICATION

SPÉCIFICATION TECHNIQUE

TECHNISCHE SPEZIFIKATION

CEN/TS 16827-1

August 2015

ICS 11.100.10

English Version

Molecular in vitro diagnostic examinations - Specifications for pre-examination processes for FFPE tissue - Part 1: Isolated RNA

Tests de diagnostic moléculaire in vitro - Spécifications relatives aux processus préanalytiques pour les tissus FFPE - Partie 1: ARN extrait

Molekularanalytische in-vitro-diagnostische Verfahren - Spezifikationen für präanalytische Prozesse für FFPE-Gewebeproben - Teil 1: Isolierte RNS

This Technical Specification (CEN/TS) was approved by CEN on 6 July 2015 for provisional application.

The period of validity of this CEN/TS is limited initially to three years. After two years the members of CEN will be requested to submit their comments, particularly on the question whether the CEN/TS can be converted into a European Standard.

CEN members are required to announce the existence of this CEN/TS in the same way as for an EN and to make the CEN/TS available promptly at national level in an appropriate form. It is permissible to keep conflicting national standards in force (in parallel to the CEN/TS) until the final decision about the possible conversion of the CEN/TS into an EN is reached.

CEN members are the national standards bodies of Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and United Kingdom.

EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

CEN-CENELEC Management Centre: Avenue Marnix 17, B-1000 Brussels

© 2015 CEN All rights of exploitation in any form and by any means reserved worldwide for CEN national Members.

Contents

Page

European foreword

This document (CEN/TS 16827-1:2015) has been prepared by Technical Committee CEN/TC 140 "*In vitro* diagnostic medical devices", the secretariat of which is held by DIN.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN [and/or CENELEC] shall not be held responsible for identifying any or all such patent rights.

According to the CEN-CENELEC Internal Regulations, the national standards organizations of the following countries are bound to announce this Technical Specification: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

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 profile generated during the pre-examination process. Therefore, a standardization of the entire process from primary sample collection to RNA analysis is needed. Studies have been undertaken to determine the important influencing factors. This Technical Specification draws upon such work to codify and standardize the steps for formalin fixed and paraffin embedded (FFPE) tissue with regard to RNA 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 RNA analysis 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).

The formalin fixation and the paraffin embedding process lead to modifications of the RNA 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 RNA analysis.

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

3.11
RNA
ribonucleic acid
polymer of ribonucleotides occurring in a double-stranded or single-stranded form

[SOURCE: EN ISO 22174:2005, 3.1.3]

3.12
room temperature
temperature which is defined as 18 °C to 25 °C for the purposes of this document

3.13
sample
one or more parts taken from a primary sample

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

3.14
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.

3.15
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.

3.16
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 quantitative RNA profile(s) of interest should be investigated throughout the entire preanalytical workflow prior to the development and implementation of an analytical test.

Before tissues are fixed in standard buffered formalin solution, RNA profiles can change significantly depending on the duration of warm and cold ischemia and the temperature before formalin fixation (e.g., gene induction, gene down regulation, RNA degradation). In addition, those changes 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 of the tissue specimen, the higher is the risk that changes in the RNA profile can occur.

NOTE Intraoperative warm ischemia can result in more pronounced changes of RNA profiles than in postoperative cold ischemia. RNA profiles can also vary, depending on the origin and type of tissue, the underlying disease, the surgical procedure, 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 RNA extracted from FFPE tissues that should be considered in quality control and application of molecular assays, especially in the context of gene expression studies (see [1], [2], [3]). Assay optimization for FFPE tissues or the use of non-crosslinking alternatives to standard buffered formalin solution are options to minimize this issue for molecular analyses (see [4]).

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 RNA 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 RNA 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 the changes to the RNA profile.

NOTE There is evidence that RNA in tissues can be stabilized in plastic bags under vacuum when kept at 0 °C to 4 °C during transport [5] 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.

NOTE Temperature conditions during transport can influence the quantitative RNA profile and RNA quality.

6.2 Formalin fixation of the specimen

The fixative used shall 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 [3]; see also A.2.1 and A.2.2. 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 slow penetration of formaldehyde from the surface of the tissue to the interior.

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 fixation progression. There is evidence that a ratio of 10:1 can be necessary [7]. 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 RNA 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 RNA 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 shall 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 transferred 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 it is essential that water be extracted from the tissue sample and replaced with paraffin wax. It is important to replace water quantitatively, since residual water leads to tissue degradation during storage [7].

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 and temperature of each embedding step shall be

performed according to the manufacturers' instructions or laboratories' validated protocols. The applied protocol shall be documented.

6.6 Storage requirements

The storage duration and temperature, typically ambient temperature in routine archives, influence the RNA stability in FFPE tissue samples [8].

RNA degradation occurring during storage can have an impact on the validity and reliability of analytical test results.

The FFPE block should be stored dry at room temperature or preferably at lower temperature.

NOTE 1 Lower storage temperatures (e.g., +4 °C, -20 °C) slow down the RNA degradation process over time [8]. See also A.2.4.

NOTE 2 If the FFPE blocks are not stored dry, the RNA degradation can increase and fungi can grow.

For RNA extractions, FFPE sections should be freshly prepared. If storage of these sections cannot be avoided, they should be stored for as short a duration as possible, dry, refrigerated (at 2 °C to 8 °C) or at lower temperatures.

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

6.7 Isolation of the total RNA

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 RNA isolation procedures

If RNA is extracted from archived tissue blocks, the blocks should be trimmed before taking the sections for RNA isolation, as the outer sections may contain degraded RNA.

Formalin fixation introduces covalent modifications to the RNA by addition of mono-methylol groups. In a second step electrophilic addition of N-methylol on an amino base leads to the formation of methylene bridges between two amino groups [2]. Fixation leads to crosslinking between RNA and proteins. Formaldehyde introduces chemical modifications interfering with enzymatic downstream analysis such as RT-PCR.

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 [9]. Prolonged tissue fixation results in enhanced RNA modifications and should be avoided.

2. Starting material for RNA 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 RNA purification. Where RNA purification has to be performed with stained material, the staining solution should be freshly prepared, using RNase-free reagents to minimize the impact of the staining on RNA quality.

NOTE Staining can impair RNA quality and performance in later (downstream) analytical applications.

4. The DNase, other reagents and consumables coming in touch with the sample shall be RNase-free.

5. The RNA isolation performance should be tested in a RNA proficiency test program.

6. For all RNA isolation procedures, measures (e.g. proteinase K digestion and heating) have to be included to reverse the formaldehyde modifications such as nucleic acids and protein-RNA crosslinks, without further RNA degradation.

The laboratory shall validate the process from the isolation of RNA 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 RNA 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.

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,

RNA isolation procedures should contain the following steps:

1. Removal of paraffin from freshly cut FFPE tissue sections by treating with xylene or xylene substitutes, centrifugation, followed by washing of the resulting pellet with ethanol.

   NOTE Commercially available deparaffinization solutions can be used, obviating the need to pellet FFPE samples.

2. Resuspension of the sections in a lysis buffer, followed by digestion with proteinase K to remove cross-linked proteins and release the RNA from the sections. Where a heating step is included into the protocol, the lysis buffer should not contain high concentrations of chaotropic salts. The combination of high salt and elevated temperatures can lead to RNA degradation

   NOTE 1 The typical incubation duration can range from 10 min to 18 h (depending on the sample type and size) at a temperature varying from 37 °C to 60 °C.

   NOTE 2 It is advised to optimize lysis buffer for the proteinase digestion step. An example can be found in [2].

3. The extraction of RNA from the lysate can be performed by mono- or biphasic-extraction, such as phenol/chloroform-based procedures, or by solid phase absorption, such as silica bead procedures.

4. A DNase treatment step or other measures to minimize DNA content in the isolated RNA should be incorporated into the RNA isolation procedure. The DNase, other reagents and consumables in contact with the sample shall be RNase-free.

6.8 Quantity and quality assessment of isolated RNA

The RNA quantity and quality should be checked according to the diagnostic kit manufacturer´s instructions, or according to validated procedures by generally accepted physical, chemical and biochemical procedures [2], [10], [11]. These may include one or more of the following:

a) quantification by absorption (A_{260}) or spectrofluorometry;

b) test for purity by absorption measurements (wavelength scan, A_{260}/A_{280} ratio);

c) test for RNA integrity and amplifiability (by electrophoresis, chromatography, or molecular methods such as the 3´/5´assay or differential length amplicon ratio [3]);

d) test for presence of interfering substances (using exogenous controls (spiked in RNA and DNA controls) or inspecting qPCR response curves for anomalies).

NOTE 1 For qualitative analyses, such as presence/absence, sequencing, 6.8, a) and b) are sufficient; for quantitative analyses, such as gene expression analysis 6.8, a) to d) are required.

NOTE 2 Formalin fixation has a negative impact on RNA integrity and reliability of RNA quality measurements. Chemical modifications caused by formaldehyde cannot be reliably detected in standard quality control assays such as electrophoretic RNA fragment length measurement, but they interfere with enzymatic analysis.

6.9 Storage of isolated RNA

The RNA isolation kit provider’s specific instructions for storing and archiving the isolated RNA shall be followed.

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

NOTE 1 Depending on the RNA isolation procedure and the resulting eluate quality, storage on wet-ice for a short period of time (e.g., 1 h) can be feasible.

Storage for archiving purposes should be at -70 °C or below; see also A.2.4.

NOTE 2 Some RNA isolation procedures can allow storing the RNA at -20 °C to -70 °C.

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

For archiving, aliquots of the isolated RNA should be generated to avoid freezing and thawing. The aliquots should not be further diluted to avoid a reduction of the RNA quality.

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 Summary

RNA from different formalin fixed and paraffin embedded (FFPE) human liver samples was used to assess the impact of formalin fixation, fixation time and storage of tissue blocks on downstream reactions such as complementary DNA (cDNA) synthesis and reverse transcription quantitative polymerase chain reaction (RT-qPCR) and its implications for quality control in comparison with snap frozen samples which served as reference. The data revealed that formalin fixation led to less efficient cDNA synthesis and RT-qPCR reactions and introduced major gene transcript to gene transcript variations. These differences could not be reliably detected by quality control using routine electrophoresis or spectrophotometry based methods but by using an RT-qPCR assay based on different amplicon length and a cDNA generation efficiency assay. Furthermore, RNA degradation depending on storage conditions and time was observed in human samples and by using an animal model.

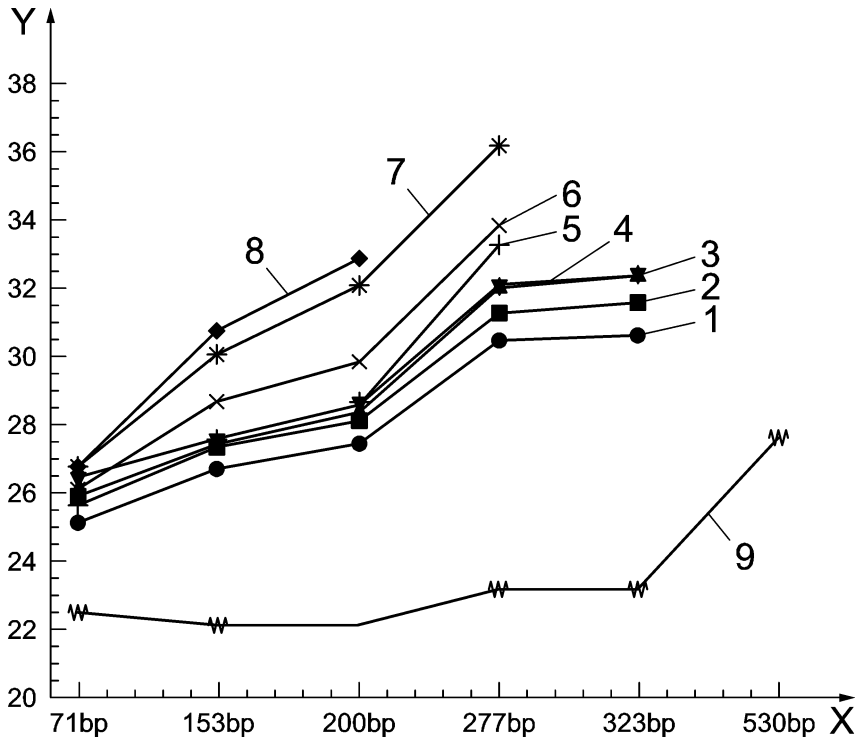
A.2 Results

A.2.1 Time dependency of RNA integrity

Formalin fixation impairs RNA integrity in a time dependent manner as shown in Figure A.1 and Figure A.2.

Multiple aliquots of a human liver sample were fixed for different time periods ranging from 4 h to 120 h in standard buffered formalin solution before paraffin embedding. Similar RNA Integrity Number (RIN) values were obtained for all fixation time points ranging from 2,1 to 2,7. However, the RT-qPCR amplification of fragments of different length of the housekeeping gene GAPDH revealed a rise in cycle threshold (c_t) values for different amplicons correlating with the fixation time. Prolonged fixation induced a steeper slope of the generated curves indicating increased fragmentation and made the amplification of longer fragments impossible.

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



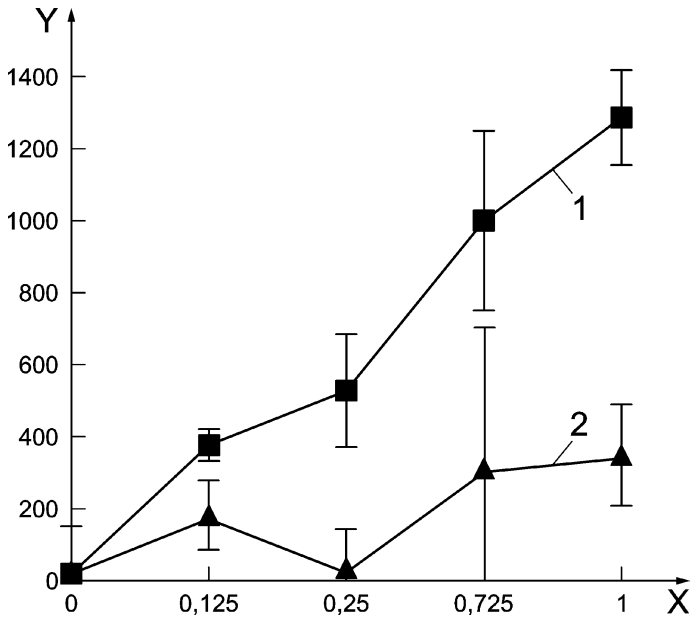
Key

X	amplicon length in bp	3	12 h	7	96 h
Y	c_t value (threshold = 0,2)	4	24 h	8	120 h
1	4 h	5	48 h	9	snap frozen tissue sample
2	8 h	6	72 h		

Figure A.1 — RT-qPCR amplification of GAPDH fragments of different length for human liver samples fixed in standard buffered formalin solution for different time periods before paraffin embedding

A.2.2 Impact of formalin-fixation on cDNA synthesis efficiency

The cDNA generation of RNA extracted from snap frozen human liver tissue (see Figure A.2) was in direct correlation to the amount of template RNA. Such correlation is wanted. RNA extracted from FFPE tissue (see Figure A.2) produced only small amounts of cDNA even when more template RNA was provided. The error bars in Figure A.2 depict the standard deviation of the median result in three individual cDNA preparations.

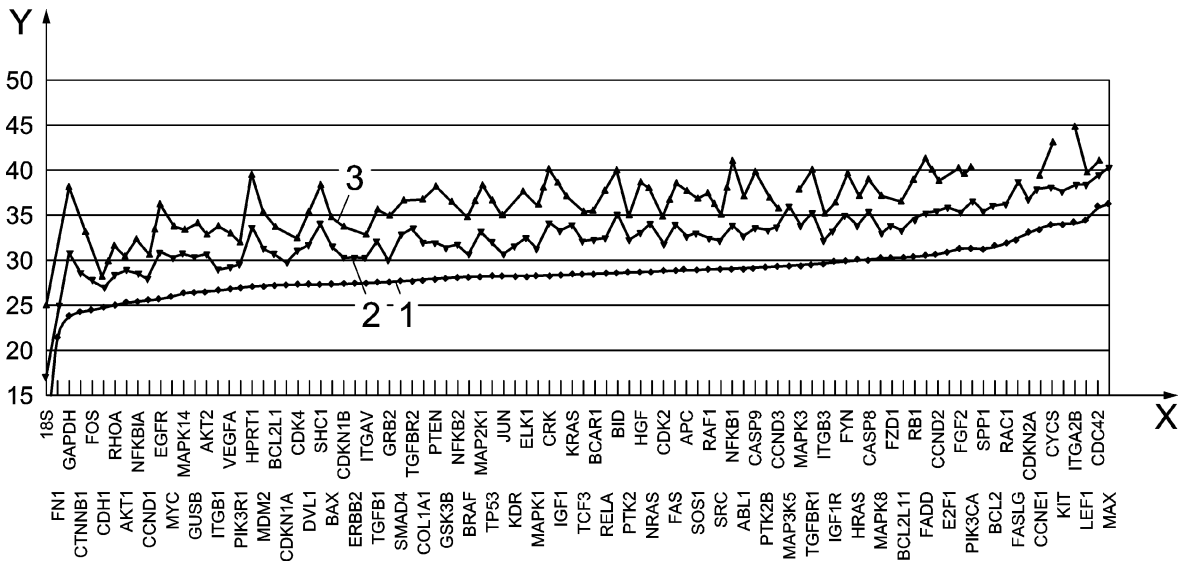


Key

X	template RNA in µg	1	snap frozen liver tissue sample
Y	relative amount of cDNA	2	FFPE liver tissue sample

Figure A.2 — generation of extracted cDNA from snap frozen and FFPE human liver tissue samples in correlation to the amount of template RNA

A.2.3 Fixation and storage introduces major gene-to-gene variations in RT-qPCR



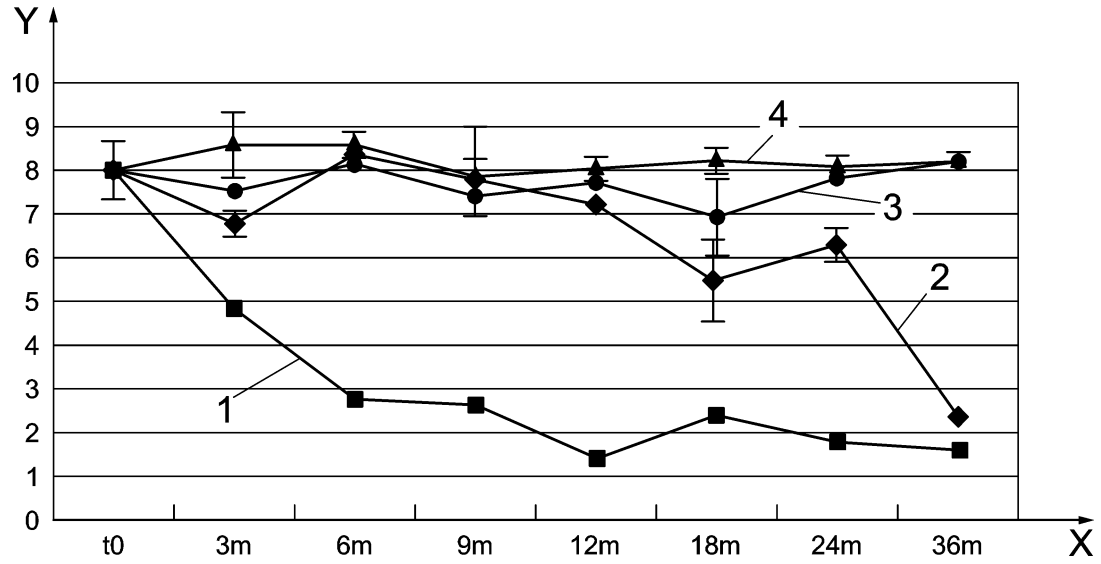
Key

X	different tested genes	1	snap frozen liver tissue sample
Y	c_t values	2	FFPE liver tissue sample after 6 months
		3	FFPE liver tissue sample after 1 year

Figure A.3 — c_t values for 92 genes from snap frozen and FFPE human liver tissue

As depicted in Figure A.3, aliquots of a human liver sample were snap frozen or fixed in standard buffered formalin solution and paraffin embedded. The RNA was extracted from tissue samples at different time points (after 6 months and 1 year). Comparison of RT-qPCR data for 92 genes from snap frozen and FFPE human liver tissue samples revealed an average difference of the c_t values ranging from 4 cycles (6 months) to 8 cycles (1 year) increasing with storage time at room temperature. Furthermore, major transcript to gene transcript variations were observed in RNA from all FFPE samples as compared to the snap frozen reference sample. This different and gene-specific behaviour of RNA extracted from FFPE samples could severely impact on the results and interpretation of gene expression studies.

A.2.4 Impact of storage conditions of FFPE blocks on RNA Integrity



Key

X	months (m)	2	storage temperature 4 °C
Y	RIN value	3	storage temperature -20 °C
t_0	beginning of storage	4	storage temperature -80 °C
1	storage temperature 22 °C		

Figure A.4 — RIN values of RNA extracted from FFPE rat spleen tissue samples stored at different temperatures

For the analysis of RIN values of RNA from FFPE rat tissue samples FFPE tissue blocks were stored prior to RNA extraction at different temperatures (22 °C, 4 °C, -20 °C, and -80 °C). RNA was extracted at beginning of storage (t_0) and after 3, 6, 9, 12, 18, 24 and 36 months of storage (see Figure A.4).

RIN values are shown for triplicate extractions from spleen tissue for each storage temperature. A constant decrease of the RIN value was observed in blocks stored at 22 °C. Degradation was slowed down in blocks stored at 4 °C. In RNA from frozen blocks (-20 °C and -80 °C) no major degradation was observed by RIN analysis.

A.3 Conclusions

Chemical modifications on RNA introduced by formalin fixation have an impact on downstream analyses like reverse transcription followed by polymerase chain reaction (RT-PCR). In case of RT-PCR this impact can be measured as decreased amplification and cDNA synthesis efficiency compared to chemically unmodified RNA from e.g., snap frozen tissue.

A standardization of the preanalytical workflow, application of additional quality control assays and detailed sample information can markedly improve the comparability and reliability of molecular studies based on formalin fixed and paraffin embedded tissue samples.

A.4 Further reading

Kashofer K, Viertler C, Pichler M, Zatloukal K (2013) Quality Control of RNA Preservation and Extraction from Paraffin-Embedded Tissue: Implications for RT-PCR and Microarray Analysis. PLoS ONE 8(7): e70714. doi:10.1371/journal.pone.0070714

www.spidia.eu

Bibliography

[1] KRAFFT D., BIJWAARD T. Lichy. Optimization of the Isolation and Amplification of RNA From Formalin-fixed, Paraffin-embedded Tissue: The Armed Forces Institute of Pathology Experience and Literature Review. *Mol. Diagn.* 1997, **2** pp. 217–230. DOI:10.1054/MODI00200217

[2] MASUDA N., OHNISHI T., KAWAMOTO S., MONDEN M., OKUBO K. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. *Nucleic Acids Res.* 1999, **27** pp. 4436–4443

[3] Kashofer K., Viertler C., Pichler M., Zatloukal K. Quality control of RNA preservation and extraction from paraffin-embedded tissue: Implications for RT-PCR and microarray analysis. PLoS One. 2013 Jul 31;8(7):e70714. doi: 10.1371/journal.pone.0070714. Print 2013.

[4] Viertler C., Groelz D., Gündisch S., Kashofer K., Reischauer B., Riegman P.H. et al. A new technology for stabilization of biomolecules in tissues for combined histological and molecular analyses. J. Mol. Diagn. 2012 Sep, 14 (5) pp. 458–466 Epub 2012 Jun 28. DOI:10.1016/j.jmoldx.2012.05.002

[5] DI NOVI C., MINNITI D., BARBARO S. et al. Vacuum-based preservation of surgical specimens: an environmentally safe step towards a formalin-free hospital. *Sci. Total Environ.* 2010, **408** (16) pp. 3092–3095

[6] FOX C.H., JOHNSON F.B., WHITING J., ROLLER P.P. Formaldehyde Fixation. *J. Histochem. Cytochem.* 1985, **33** pp. 845–853

[7] 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. *Arch. Pathol. Lab. Med.* 2008, **132** pp. 1929–1935

[8] VON AHLFEN S., MISSEL A., BENDRAT K., SCHLUMPBERGER M. Determinants of RNA Quality from FFPE Samples. *PLoS ONE*. 2007, **2** (12) p. e1261

[9] CHUNG J.Y., BRAUNSCHWEIG T., WILLIAMS R., GUERRERO N., HOFFMANN K.M., KWON M. et al. Factors in tissue handling and processing that impact RNA obtained from formalin-fixed, paraffin-embedded tissue. *J. Histochem. Cytochem.* 2008, **56** pp. 1033–1042

[10] GROELZ D., SOBIN L., BRANTON P., COMPTON C., WYRICH R., RAINEN L. Non-formalin fixative versus formalin-fixed tissue: A comparison of histology and RNA quality. *Exp. Mol. Pathol.* 2013, **94** pp. 188–194

[11] KUBISTA M., BJORKMAN J., SVEC D., SJOBACK R. RNA quality matters. *European Pharmaceutical Reviews.* 2012, **17** (6) pp. 1–5

[12] LIBUS J., STORCHOVA´ H. Quantification of cDNA generated by reverse transcription of total RNA provides a simple alternative tool for quantitative RT-PCR normalization. *Biotechniques*. 2006, **41** pp. 156, 158, 160 passim

[13] EN ISO 22174:2005, *Microbiology of food and animal feeding stuffs - Polymerase chain reaction (PCR) for the detection of food-borne pathogens - General requirements and definitions (ISO 22174:2005)*

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

This page deliberately left blank

NO COPYING WITHOUT BSI PERMISSION EXCEPT AS PERMITTED BY COPYRIGHT LAW

British Standards Institution (BSI)

BSI is the national body responsible for preparing British Standards and other standards-related publications, information and services.

BSI is incorporated by Royal Charter. British Standards and other standardization products are published by BSI Standards Limited.

About us

We bring together business, industry, government, consumers, innovators and others to shape their combined experience and expertise into standards -based solutions.

The knowledge embodied in our standards has been carefully assembled in a dependable format and refined through our open consultation process. Organizations of all sizes and across all sectors choose standards to help them achieve their goals.

Information on standards

We can provide you with the knowledge that your organization needs to succeed. Find out more about British Standards by visiting our website at bsigroup.com/standards or contacting our Customer Services team or Knowledge Centre.

Buying standards

You can buy and download PDF versions of BSI publications, including British and adopted European and international standards, through our website at bsigroup.com/shop, where hard copies can also be purchased.

If you need international and foreign standards from other Standards Development Organizations, hard copies can be ordered from our Customer Services team.

Subscriptions

Our range of subscription services are designed to make using standards easier for you. For further information on our subscription products go to bsigroup.com/subscriptions.

With **British Standards Online (BSOL)** you'll have instant access to over 55,000 British and adopted European and international standards from your desktop. It's available 24/7 and is refreshed daily so you'll always be up to date.

You can keep in touch with standards developments and receive substantial discounts on the purchase price of standards, both in single copy and subscription format, by becoming a **BSI Subscribing Member**.

PLUS is an updating service exclusive to BSI Subscribing Members. You will automatically receive the latest hard copy of your standards when they're revised or replaced.

To find out more about becoming a BSI Subscribing Member and the benefits of membership, please visit bsigroup.com/shop.

With a **Multi-User Network Licence (MUNL)** you are able to host standards publications on your intranet. Licences can cover as few or as many users as you wish. With updates supplied as soon as they're available, you can be sure your documentation is current. For further information, email bsmusales@bsigroup.com.

BSI Group Headquarters

389 Chiswick High Road London W4 4AL UK

Revisions

Our British Standards and other publications are updated by amendment or revision.

We continually improve the quality of our products and services to benefit your business. If you find an inaccuracy or ambiguity within a British Standard or other BSI publication please inform the Knowledge Centre.

Copyright

All the data, software and documentation set out in all British Standards and other BSI publications are the property of and copyrighted by BSI, or some person or entity that owns copyright in the information used (such as the international standardization bodies) and has formally licensed such information to BSI for commercial publication and use. Except as permitted under the Copyright, Designs and Patents Act 1988 no extract may be reproduced, stored in a retrieval system or transmitted in any form or by any means – electronic, photocopying, recording or otherwise – without prior written permission from BSI. Details and advice can be obtained from the Copyright & Licensing Department.

Useful Contacts:

Customer Services
Tel: +44 845 086 9001
Email (orders): orders@bsigroup.com
Email (enquiries): cservices@bsigroup.com

Subscriptions
Tel: +44 845 086 9001
Email: subscriptions@bsigroup.com

Knowledge Centre
Tel: +44 20 8996 7004
Email: knowledgecentre@bsigroup.com

Copyright & Licensing
Tel: +44 20 8996 7070
Email: copyright@bsigroup.com

bsi.

...making excellence a habit.™