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BSI Standards Publication

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

Part 1: Isolated RNA

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National foreword

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

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

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Contents

Page

European foreword

This document (CEN/TS 16826-1:2015) 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 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 frozen 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 frozen 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 organisations performing biomedical research, biobanks, and regulatory authorities).

RNA profiles in tissues can change significantly before and after collection and can change differently in tissues from different donors / patients.

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

Tissues that have undergone chemical stabilisation pre-treatment before freezing are not covered in this document.

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
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.

3.5
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.]

3.6
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

3.7
RNA
ribonucleic acid
polymer of ribonucleotides occurring in a double-stranded or single-stranded form

[SOURCE: EN ISO 22174:2005, 3.1.3]

3.8
room temperature
temperature which is defined as 18 °C to 25 °C for the purposes of this document

3.9
sample
one or more parts taken from a primary sample

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

3.10
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.

3.11
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 stabilized by freezing, quantitative RNA profile can change e.g., by gene induction, gene down regulation and RNA degradation. These effects depend on the duration of warm and cold ischemia and the ambient temperature before freezing. In addition, the described effects can vary in tissues from different donors / patients.

Generally, the longer the warm and cold ischemia times and the higher the ambient temperature before freezing 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 during postoperative cold ischemia. RNA profiles can also vary, depending on the origin and type of tissue, the underlying disease, the surgical procedure, the drug regime, and drugs administered for anaesthesia or treatment of concomitant disease and on the different environmental conditions after the tissue removal from the body.

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

Safety regulations on transport and handling shall be 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 6.3, if freezing is performed 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);

3. the selection and use of stabilisation 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) will lead to RNA degradation when the tissue thaws thereafter. It can also impact the morphological characterisation.

   NOTE 2 This step can be omitted, if the specimen is directly frozen (see 6.3.)

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 frozen, it should be transported on wet-ice or at 2 °C to 8 °C without delay in order to minimize changes to the RNA profile.

NOTE There is evidence that RNA in tissues can be stabilised in plastic bags under vacuum when kept at 0 °C to 4 °C during transport [1] before the samples are archived for biobanks or used for histopathological evaluation.

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 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 primary sample 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 freezing 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 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 without the requirement of histopathological diagnosis; the evaluation, documentation and selection of samples may be done by other qualified persons than pathologists.

c) Where frozen section diagnosis is needed, the selected part of the specimen shall be frozen in an appropriate freezing medium. Frozen sections shall be evaluated by a medically qualified (e.g., board certified) pathologist. The remaining sample frozen in the freezing medium can be stored or further processed for RNA analysis. In this case the frozen section made for diagnostic purposes can be used for characterisation of the sample's cellular composition, disease condition and preservation status.

6.3 Cryo-storage of the specimen

The following steps shall be performed:

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

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

3. the consideration of the tissue size, which determines the size of the container:

    a) the sample should not exceed 1 cm in one dimension;

4. the selection of the sample container for cryo-storage:

    a) the container shall have the right volume for the size of the specimen to be stored in it;

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

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

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

    d) the containers shall be suited for permanent labelling under frozen storage conditions;

5. the labelling shall be suitable for the respective frozen storage conditions;

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

6. the labelling of the cryo-storage container shall contain 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 and disease condition such as affected (e.g., tumour) or unaffected, unless a sample tracking system can supply this information coupled to the identification of the sample used in a);

    c) the unique numbering of each cryo-container;

7. the documentation of types and quantity and description of samples:

    a) 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.

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 aspect of a disease is actually reflected in the tissue sample used for molecular analysis (e.g., different molecular mechanisms can be activated at the centre or the invasion front of the tumour, also tumours can be composed of areas showing variations in differentiation grades).

6.4 Storage requirements

The constant temperature shall be below -70 °C. Systems monitoring the temperature should be used.

Major temperature shifts may occur during retrieval of samples. Therefore, retrieval times should be kept as short as possible for avoiding the thawing of samples.

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

Freezers shall have a temperature alarm system.

Back-up cryo-storage facilities should be provided.

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

6.5 Isolation of the total RNA

6.5.1 General information for RNA isolation procedures

Freezing of tissue may result in the disruption of cellular membranes and organelles within the tissue. As a consequence, after thawing enzymes may be released and activated which may lead to degradation of RNA.

Therefore, the sample shall not thaw before its homogenous dispersion in lysis buffers containing RNase inhibiting substances. The tissue sample should be thoroughly minced or cut into small pieces in its frozen state and thoroughly dispersed with lysis buffers containing RNase inhibiting substances. The subsequent homogenisation of the frozen tissue sample in the lysis buffer shall be processed immediately after having introduced the tissue into this lysis buffer.

This step has major impact on the stabilisation of the RNA integrity and yield and should be controlled in the laboratory. Suitable homogenisation devices for isolation of RNA from tissues are commercially available.

Alternatively, sections that have been cut on a cryotome (thickness of 4 µm to 20 µm) shall be submerged, while still frozen, directly into the lysis buffers containing RNase inhibiting substances.

Requirements and recommendations:

1. All materials (excluding the lysis buffer and vial containing this buffer) and tools used to manipulate the frozen sample for cryo-sectioning or transferring to the lysis buffer shall be sterile to minimize contamination with nucleases and should be cooled to at least -20 °C before use.

2. Where morphology changes (e.g., tumour content) can influence the analysis of the results, it is strongly recommended to use cryo-sections for RNA isolation and check the morphology after every 50 µm by cutting a section for Haematoxylin and Eosin (H&E) staining. Where a histopathological characterisation of the cellular composition and disease condition of the sample was not performed under 6.2, it shall be performed at this stage to assess the cellular composition and disease condition. It can also serve as reference to proof, if the disease state is still present in the required percentage or not in the sample intended to be analysed.

3. The incorporation of a DNase treatment step into the RNA isolation procedure is recommended. The DNase, other reagents and consumables coming in touch with the sample shall be RNase-free.

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

The extracted RNA should be kept on wet-ice or at 2 °C to 8 °C (e.g., cooling block) and should be assayed immediately.

To avoid a cross contamination with amplified material from the RNA analytical test, the isolation of the RNA should not be performed in the same area as the amplification, unless a closed system is used.

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).

6.5.2 Using commercial kits

When using commercial kits for the isolation of RNA from frozen tissues, the manufacturers' instructions for use shall be followed.

6.5.3 Using the laboratories' own protocols

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

6.5.3.2 If the laboratory uses its own protocol 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.

6.6 Quality assessment of isolated RNA

The RNA quantity and quality should be checked according to diagnostic kit manufacturer´s instructions, or according to validated procedures by generally accepted physical, chemical and biochemical procedures [2], [3]. 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 (by electrophoresis, chromatography, or molecular methods such as the 3´/5´assay or differential length amplicon ratio [4]);

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

NOTE For qualitative analyses, such as presence/absence, sequencing, 6.6, a) and b) are sufficient; for quantitative analyses, such as gene expression analysis 6.6, a) to d) are required.

6.7 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 RNA cannot be assayed immediately, the laboratory shall have verified procedures in place how to store the isolated RNA.

NOTE 1 Depending on the RNA isolation procedure 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.

NOTE 2 Some RNA isolation procedures may 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)

Impact of preanalytical variables on RNA profiles obtained from frozen liver tissue samples collected during and after routine surgery

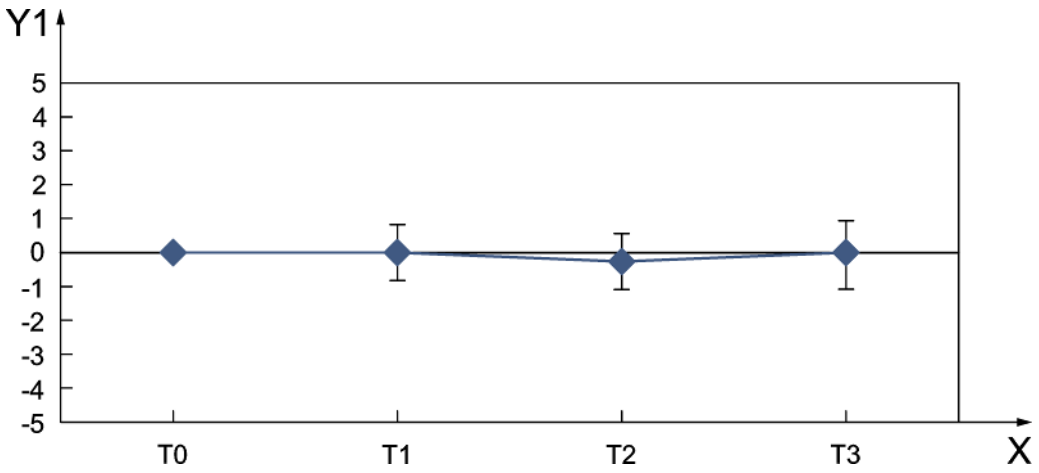
A.1 Comparison of stable and unstable genes identified under ischemic conditions

Within the EU FP7 SPIDIA project[1)] a comprehensive multicentre study was performed to identify changes of RNA profiles by preanalytical variation in tissue samples. Non-malignant tissue samples were collected at different time points during and after routine liver surgery and snap frozen in isopentane (methyl butane) cooled by and stored in liquid nitrogen until further analysis.

RNA was extracted and microarray analysis of all ischemia samples was performed. Genes were selected which showed RNA profile changes due to preanalytical variation. These changes were further validated by RT-qPCR.

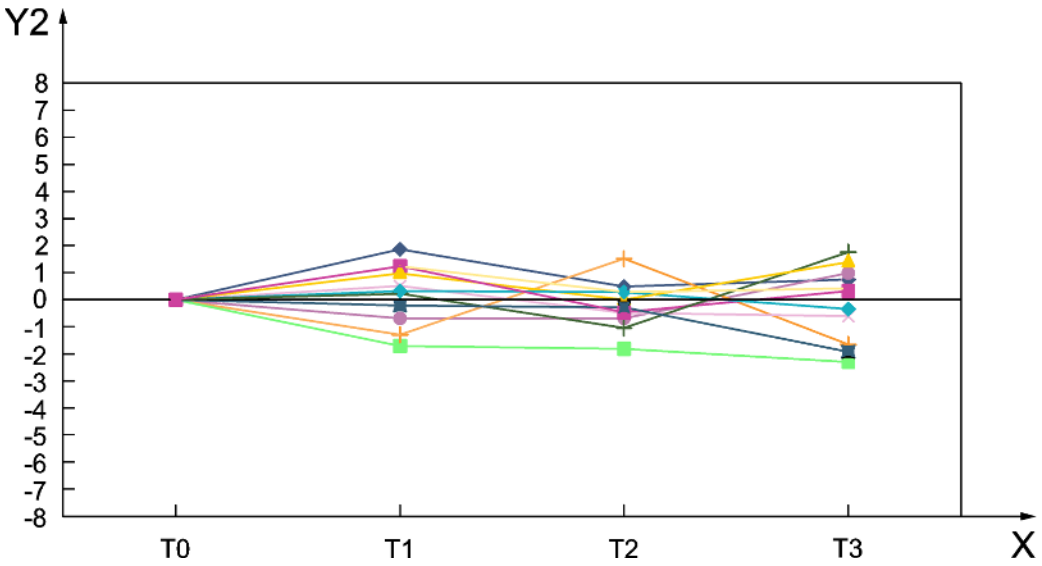
It was observed that some RNA profiles reacted differently from patient to patient under ischemic conditions, as displayed in Figure A.2 in comparison with a stable RNA profile (Figure A.1) identified in the study. These unpredictable variations could cause major problems, e.g., in biomarker discovery and development. Hence, the preanalytical workflow should be carefully evaluated and documented.

During the design of the analytical test system it is strongly recommended to investigate, if the specific RNA profile/s intended to be analysed in the analytical test is/are not affected by the entire preanalytical workflow.



a) mean delta quantification cycles at different time points

1) Research by the EU FP7 SPIDIA project funded by the European Union Seventh Framework Programme [FP7/2007-2013] under grant agreement no 222916. For further information see www.spidia.eu.



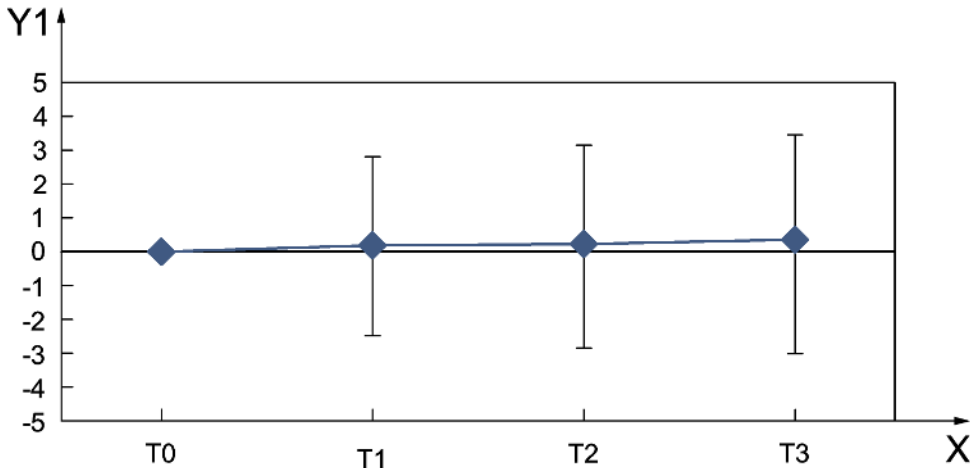
b) delta quantification cycles at different time points

Key

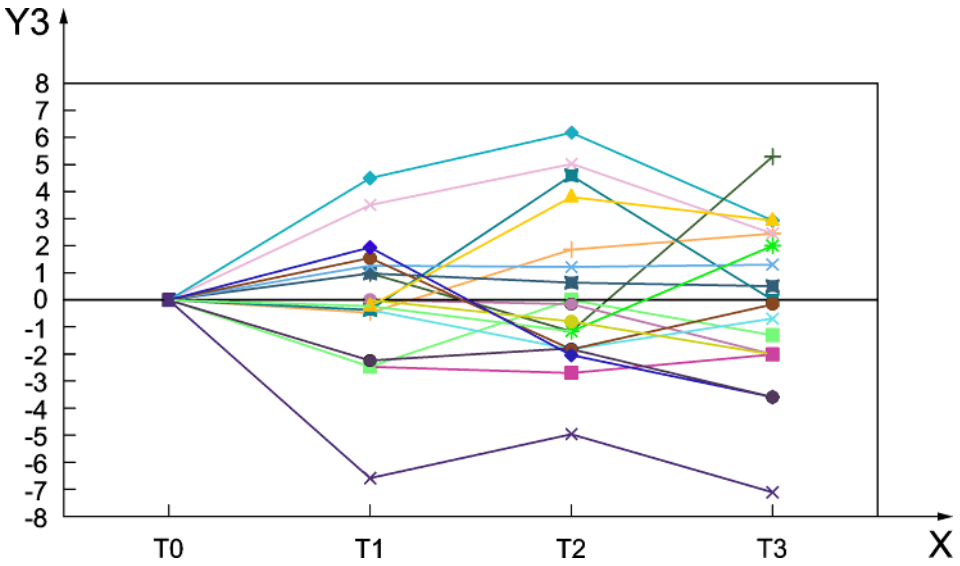
Y1	Δ*cq* mean (mean delta quantification cycle)	T1	first time point during surgery
Y2	Δ*cq*	T2	second time point during surgery
T0	reference time point at the beginning of surgery (no ischemia control)	T3	time point after tissue removal including a short cold ischemia time (usual biobanking time point)
		X	time point

NOTE The results from T1 to T3 were normalized to the T0 time points to receive the Δ*cq* values.

Figure A.1 —Example of a stable RNA profile (A) in different donors under ischemic conditions



a) mean delta quantification cycles at different time points



b) delta quantification cycles at different time points

Key

Y1	Δ*cq* mean (mean delta quantification cycle)	T1	first time point during surgery
Y3	Δ*cq*	T2	second time point during surgery
T0	reference time point at the beginning of surgery (no ischemia control)	T3	time point after tissue removal including a short cold ischemia time (usual biobanking time point)
		X	time point

NOTE The results from T1 to T3 were normalized to the T0 time points to receive the Δ*cq* values.

Figure A.2 — Example of an unstable RNA profile (B) in different donors under ischemic conditions

Although the mean delta quantification cycles (Δ*cq* values) appear stable over all time points for RNA profile A (Figure A.1, a)) and RNA profile B (Figure A.2, a)), the standard deviations and individual patient results (Figure A.1, b); Figure A.2, b)) revealed high inter-patient variability for RNA profile B as compared to the stable RNA profile A. Therefore, RNA profile B was considered as being unstable depending on the preanalytical ischemia conditions.

A.2 Recommendations based on the results

The experiment shows that RNA profiles can become unstable during and after the surgical procedure. This effect is only observed in a small percentage of individual RNA profiles, some of which play a role in oncogenesis (data not shown). Many variables can influence RNA profiles during the surgical procedure, for instance ischemia-relevant vessel ligation/clamping causing a warm ischemic period and after removal of the tissue from the body a cold ischemic period starts. Even the genetic background of individual donors can be of importance. Since these variables cannot be standardised or avoided, it is important to document these preanalytical parameters as described in this document.

Such parameters can be used to correlate later findings in the analytical test or to select samples in such a way that the suspected influential parameters are the same. This way, cohorts can become fit for purpose for very sensitive analytical tests involving genes that are known to be instable during the pre-acquisition phase and/or afterwards. This data can be documented in the hospital information system and it is imperative for primary sample collection managers to have access to this data and couple it to the archival data.

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