
**Molecular in vitro diagnostic
examinations — Specifications for
pre-examination processes for venous
whole blood —**

**Part 1:
Isolated cellular RNA**

*Analyses de diagnostic moléculaire in vitro — Spécifications relatives
aux processus préanalytiques pour le sang total veineux —*

Partie 1: ARN cellulaire extrait



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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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

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

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 212, *Clinical laboratory testing and in vitro diagnostic test systems*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

A list of all parts in the ISO 20186 series can be found on the ISO website.

Introduction

Molecular in vitro diagnostics has enabled significant progress in medicine. Further progress is expected by new technologies analysing profiles of nucleic acids, proteins, and metabolites in human tissues and body fluids. However, the profiles of these molecules can change drastically during the pre-examination process, including the specimen collection, transport, storage, and processing. Consequently, this makes the outcome from diagnostics or research unreliable or even impossible, because the subsequent examination might not determine the real situation in the patient but an artificial profile generated during the pre-examination process.

Blood cellular RNA profiles can change significantly after blood collection. Therefore, special measures need to be taken to secure good quality blood samples for cellular RNA examination and storage.

Standardization of the entire workflow from specimen collection to the cellular RNA examination is needed. Studies have been undertaken to determine the important influencing factors. This document draws upon such work to codify and standardize the steps for venous whole blood cellular RNA examination in what is referred to as the pre-examination phase.

In this document, the following verbal forms are used:

- “shall” indicates a requirement;
- “should” indicates a recommendation;
- “may” indicates a permission;
- “can” indicates a possibility or a capability.

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Molecular in vitro diagnostic examinations — Specifications for pre-examination processes for venous whole blood —

Part 1: Isolated cellular RNA

1 Scope

This document gives guidelines on the handling, storage, processing and documentation of venous whole blood specimens intended for cellular RNA examination during the pre-examination phase before a molecular examination is performed. This document covers specimens collected in venous whole blood collection tubes.

This document is applicable to any molecular in vitro diagnostic examination performed by medical laboratories. It is also intended to be used by laboratory customers, in vitro diagnostics developers and manufacturers, biobanks, institutions and commercial organizations performing biomedical research, and regulatory authorities.

Different dedicated measures are taken for stabilizing blood cell free circulating RNA and RNA in exosomes circulating in blood. These are not described in this document.

Different dedicated measures are taken for collecting, stabilizing, transporting and storing capillary blood as well as for collecting and storing blood by paper based technologies or other technologies generating dried blood. These are not described in this document.

This document does not cover the isolation of specific blood cells and subsequent isolation of cellular RNA therefrom.

RNA in pathogens present in blood is not covered by this document.

2 Normative references

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

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

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

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

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <http://www.electropedia.org/>

3.1

ambient temperature

unregulated temperature of the surrounding air

3.2

analyte

component represented in the name of a measurable quantity

[SOURCE: ISO 17511:2003, 3.2]

3.3

backflow

flow of a liquid opposite to the usual or desired direction

3.4

blood cellular RNA

cellular RNA

RNA molecules present in blood cells

3.5

blood cellular RNA profile

amounts of different RNA molecules, that are present in blood cells and that can be measured in the absence of any losses, inhibition and interference

3.6

blood cellular RNA profile stabilizers

compounds, solutions or mixtures that are designed to minimize changes of the *blood cellular RNA profile* (3.5)

3.7

blood collection set

intravenous device specialized for venepuncture consisting of a stainless steel beveled needle and tube (tubing) with attached plastic wings and fitting connector

Note 1 to entry: The connector attaches to an additional blood collection device, such as a *blood collection tube* (3.8).

3.8

blood collection tube

tube used for blood collection, usually in a vacuum which forces blood from the vein through the needle into the tube

3.9

closed system

non-modifiable system provided by the vendor including all necessary components for the examination (i.e. hardware, software, procedures and reagents)

3.10

deoxyribonucleic acid

DNA

polymer of deoxyribonucleotides occurring in a double-stranded (dsDNA) or single-stranded (ssDNA) form

[SOURCE: ISO 22174:2005, 3.1.2]

3.11

deoxyribonuclease

DNase

enzyme that catalyzes the degradation of DNA into smaller components

3.12**examination
analytical test**

set of operations having the object of determining the value or characteristics of a property

[SOURCE: ISO 15189:2012, 3.7, modified — Term and definition are used here without the original notes; an additional term was added.]

Note 1 to entry: Processes that start with the isolated *analyte* (3.2) and include all kinds of parameter testing or chemical manipulation for quantitative or qualitative examination.

3.13**examination performance
analytical test performance
analytical performance**

ability of an examination procedure to measure or detect a particular *analyte* (3.2)

Note 1 to entry: Analytical performance is determined from analytical performance studies used to assess the ability of an in vitro diagnostic examination procedure to measure or detect a particular analyte.

Note 2 to entry: Analytical performance includes such characteristics as analytical sensitivity, detection limit, analytical specificity (interference and cross-reactivity), trueness, precision and linearity.

[SOURCE: ISO/TS 17822-1:2014, 3.2, modified — Two terms have been added.]

3.14**examination provider
analytical test provider**

entity that provides the specific analytical test

3.15**interfering substance**

endogenous or exogenous substances in clinical *specimens* (3.18)/*samples* (3.24) that can alter an *examination* (3.12) result

Note 1 to entry: Examples of endogenous substances are blood components and acidic polysaccharides.

Note 2 to entry: Examples of exogenous substances are talc and anticoagulant.

3.16**needle holder**

barrel used in routine venepuncture procedures to hold the *blood collection tube* (3.8) in place and to protect the phlebotomist from direct contact with blood

3.17**pre-examination processes
preanalytical phase
preanalytical workflow**

processes that start, in chronological order, from the clinician's request and include the examination request, preparation and identification of the patient, collection of the *primary sample(s)* (3.18), transportation to and within the medical laboratory, isolation of analytes, and end when the analytical examination begins

Note 1 to entry: The pre-examination phase includes preparative processes, e.g. RNA isolation procedures, which influence the outcome of the intended examination.

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

3.18

primary sample specimen

discrete portion of a body fluid, breath, hair or tissue taken for examination, study or analysis of one or more quantities or properties assumed to apply for the whole

[SOURCE: ISO 15189:2012, 3.16, modified — Notes to entry have been omitted.]

3.19

primary sample collection device

apparatus specifically intended by an IVD manufacturer to obtain, contain and preserve a body fluid or tissue for in vitro diagnostic examination

Note 1 to entry: Includes devices intended to store a specimen prior to examination.

Note 2 to entry: Includes both vacuum and non-vacuum specimen collection devices.

[SOURCE: ISO 18113-1:2009, 3.55]

3.20

proficiency testing

evaluation of participant performance against pre-established criteria by means of interlaboratory comparisons

[SOURCE: ISO 17043:2010, 3.7, modified — Notes to entry have been omitted.]

3.21

ribonucleic acid

RNA

polymer of ribonucleotides occurring in a double-stranded or single-stranded form

[SOURCE: ISO 22174:2005, 3.1.3]

3.22

ribonuclease

RNase

enzyme that catalyses the degradation of RNA into smaller components

3.23

room temperature

temperature in the range of 18 °C to 25 °C

Note 1 to entry: The definition is given for the purposes of this document. Local or national regulations can have different definitions.

3.24

sample

one or more parts taken from a *primary sample* ([3.18](#))

[SOURCE: ISO 15189:2012, 3.24, modified — The example has been omitted.]

3.25

stability

ability of a sample material, when stored under specified conditions, to maintain a stated property value within specified limits for a specified period of time

[SOURCE: ISO Guide 30:2015, 2.1.15, modified — The phrase “reference material” has been replaced by “sample material”.]

3.26**validation**

confirmation, through the provision of objective evidence, that the requirements for a specific intended use or application have been fulfilled

Note 1 to entry: The term “validated” is used to designate the corresponding status.

[SOURCE: ISO 9000:2015, 3.8.13, modified — Note 1 and Note 3 have been omitted.]

3.27**venous whole blood**

blood collected after directly puncturing a vein, usually with a needle and syringe, or other collection device

3.28**verification**

confirmation, through the provision of objective evidence, that specified requirements have been fulfilled

Note 1 to entry: The term “verified” is used to designate the corresponding status.

Note 2 to entry: Confirmation can comprise activities such as:

- performing alternative calculations;
- comparing a new design specification with a similar proven design specification;
- undertaking tests and demonstrations;
- reviewing documents prior to issue.

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

3.29**workflow**

series of activities necessary to complete a task

4 General considerations

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

All steps of a diagnostic workflow can influence the final examination result. Thus, the entire workflow, including specimen/sample storage and transport conditions, and its impact on the stability of biomolecules intended to be examined shall be verified and validated. Workflow steps which cannot always be controlled shall be documented and their impact on the examination performance shall be investigated and mitigation measures shall be established to enable the required examination performance. In these cases, risk assessment is recommended.

Cellular RNA profiles can change significantly after blood collection, for example by gene induction, gene down regulation or RNA degradation^{[8][9][10][11]}. These changes can vary individually in blood from different donors or patients^{[9][12][13][14][15]}, and can impact the validity and reliability of examination results.

Before or during the design of an examination, it should therefore be investigated and ensured that the specific blood cellular RNA profile(s) intended to be analysed is/are not compromised by the envisioned pre-examination process in a manner impacting the examination performance. This can, for example,

be done by performing time-course studies examining the post collection stability of the specific RNA profile intended to be analysed.

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

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

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

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

5 Outside the laboratory

5.1 Specimen collection

5.1.1 Information about the specimen donor/patient

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

The documentation should include, but is not limited to:

- a) the relevant health status of the specimen donor or patient [e.g. healthy, disease type, concomitant disease, demographics (e.g. age and gender)];
- b) the information about medical treatment and special treatment prior to blood collection (e.g. anaesthetics, medications, fasting status);
- c) the type and purpose of the proposed examination requested;
- d) the appropriate consent from the specimen donor/patient.

See also ISO 15189:2012, 5.4.4.

5.1.2 Selection of the venous whole blood collection tube by the laboratory

Due to the high instability of blood cellular RNA profiles in specimens collected from individual patients/donors [8][12][13][14][15] commercially available venous whole blood collection tubes containing blood cellular RNA profile stabilizers should be used [12][13][15][16][17] and their catalogue and lot number should be documented. See also [Figure A.1](#).

Blood collection tubes not containing any blood cellular RNA profile stabilizer should only be used, if the specific blood cellular RNA molecule or the blood cellular RNA profile to be examined is stable after blood draw during the entire pre-examination process (see [Figure A.2](#)) or if the requested examination allows the use of such tubes.

5.1.3 Venous whole blood specimen collection from the donor/patient and stabilization procedures

- a) The identity of the person collecting the specimen and the date and time of blood collection according to ISO 15189:2012, 5.4.4.3, f) shall be documented.
- b) For the labelling (sample/specimen identification) of the blood collection tube, a routine procedure [ISO 15189:2012, 5.4.4.3, e)] or a procedure with additional information (e.g. 2D-barcode) shall be used.

- c) Standard venepuncture technique can be used. Steps for preventing possible backflow into the donor's/patient's body can be required. The manufacturer's instructions for using the blood collection tubes shall be followed. A blood collection set and needle holder can be required when using blood cellular RNA profile stabilizer containing tubes. In this case, the instructions of the collection set and needle holder manufacturer shall be followed.

NOTE 1 There is no known specific effect of venous whole blood draw procedure on cellular RNA profiles. Routine procedures can therefore be used.

- d) Blood collection tubes shall be filled in accordance to the manufacturer's instructions and attention should be drawn to the correct positioning of the collection tube during the blood draw as well as the required blood volume.

NOTE 2 Underfilling of blood collection tubes containing cellular RNA profile stabilizers can compromise the function of the stabilizers due to an unfavourable blood to stabilizer ratio. This can in itself compromise the blood cellular RNA profile, which can impact the validity and reliability of the examination results.

- e) The blood collection tube manufacturer's instructions, for mixing or inverting the tube immediately after blood collection, shall be followed. Mixing or inverting the blood collection tube shall be done gently.

NOTE 3 Wrong and/or insufficient mixing can be one of the most important pre-examination variables. Unless additives in the blood collection tubes are homogeneously mixed with the specimen, the blood cellular RNA profile quality and quantity of individual cellular RNA molecules can be compromised, which can impact the validity and reliability of the examination results.

- f) Any tampering with and/or additions to the specimen shall be documented.

5.1.4 Information about the specimen and storage requirements at the blood collection facility

5.1.4.1 General

As blood cellular RNA profiles can change significantly after blood collection and can thereby affect the validity and reliability of the examination result^{[8][16][18][19]}, the documentation regarding the specimen shall include the date and time of blood collection^{[8][19][20][21]}.

In case of specimens for long-term storage in a biobank, it is usually not known which individual cellular RNA profiles will be examined after the long-term storage. Therefore, blood collection tubes without blood cellular RNA profile stabilizers should not be used for biobanking.

The storage conditions (duration and temperature, etc.) shall be documented.

The temporary storage duration in the blood collection facility contributes to the total duration for storage.

5.1.4.2 Using blood collection tubes with stabilizers

Blood tubes with cellular RNA profile stabilizers should be used. For storing the specimens collected in blood collection tubes with blood cellular RNA profile stabilizers, the dedicated blood collection tube manufacturer's instructions on storage conditions shall be followed (e.g. temperature, freezing and duration). Where the examination provider's instructions are more stringent, these shall be followed.

5.1.4.3 Using blood collection tubes without stabilizers

5.1.4.3.1 Blood collection tubes without blood cellular RNA profile stabilizers should only be used, if the ordered examination specifications allow the usage of such tubes. In these cases, the examination provider's instructions on storage conditions (temperature and duration, etc.) shall be followed.

5.1.4.3.2 When using blood collection tubes without blood cellular RNA profile stabilizers and no requirements on the storage conditions are available, the specimens should be transferred immediately

to 2 °C to 8 °C or on wet ice in order to minimize blood cellular RNA profile changes^{[13][19][20][21]} (see [Figure B.1](#)). The storage duration allowed at 2 °C to 8 °C or on wet ice is highly dependent on the stability of the individual blood cellular RNA profile to be analysed in the examination. This stability can vary between several minutes and over 24 h.

NOTE Under these conditions, blood cellular RNA profile changes can still occur when no blood cellular RNA profile stabilizers are used (see [Figure B.1](#)).

5.2 Transport requirements

The required transport conditions shall be documented including any deviations therefrom.

Temperature monitoring should be applied in a suitable manner.

When using blood collection tubes with blood cellular RNA profile stabilizers, the dedicated tube's manufacturer's instructions on transport conditions shall be followed (duration and temperature, etc.). Where the examination provider's instructions are more stringent, these shall be followed. The transport conditions (duration and temperature, etc.) shall be documented.

When using blood collection tubes without blood cellular RNA profile stabilizers, the examination provider's instructions on transport conditions shall be followed. This can require the documentation of transport conditions (duration and temperature, etc.).

When using blood collection tubes without blood cellular RNA profile stabilizers and no examination provider's instructions are available, the specimen should be transported at 2 °C to 8 °C or on wet ice without delay in order to minimize the blood cellular RNA profile changes^[21]. The transport conditions (duration and temperature, etc.) shall be documented.

NOTE Under these conditions, blood cellular RNA profile changes can still occur when no blood cellular RNA profile stabilizers are used (see [Figure B.1](#)).

See also ISO 15189:2012, 5.4.5.

The transport duration to the laboratory contributes to the total duration for storage.

6 Inside the laboratory

6.1 Specimen reception

The specimen reception date and time shall be documented as well as the name of the person receiving the specimen. Nonconformities of labelling, transport conditions and blood volume differences to specifications, leaking/broken tubes, etc. shall be documented.

NOTE This includes for example a note, when specimens shipped at 2 °C to 8 °C or on wet ice are not cool or transportation containers do not contain dry ice as intended by the sender.

Where there are nonconformities in labelling, transport conditions, overall storage and transport duration or blood volume that could affect the validity and reliability of the examination result, a new specimen should be obtained.

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

6.2 Storage requirements

The storage temperature and time interval between specimen receipt and sample processing for cellular RNA isolation shall be documented. Storage temperature and total storage duration shall not exceed specifications identified in [5.1.4](#) and [5.2](#).

The specimen total storage duration shall include the duration for storage at the blood collection facility (see 5.1.4), for transportation to the laboratory (see 5.2) and for further storage at the laboratory or other institutions.

The stability of specific blood cellular RNA profiles can vary between different RNA molecules, different blood donors/patients, and different blood storage and transport conditions. Any specified maximum storage duration given by the blood collection tube manufacturer or the provider of the examination shall not be exceeded. If such specifications are not available, the maximum storage duration shall be verified and generally kept to a minimum.

See also [Table 1](#).

Table 1 — Summary of storage conditions for venous whole blood collection tubes with or without blood cellular RNA profile stabilizers

Blood collection tube	Blood collection, transport and storage	
	Duration	Temperature
With blood cellular RNA profile stabilizer	— Blood collection tube manufacturer's instructions — Examination provider's instructions ^a	
Without blood cellular RNA profile stabilizer	— Examination provider's instructions	— Examination provider's instructions — Wet ice or 2 °C to 8 °C ^b
^a If more stringent than blood tube manufacturer's instructions. ^b If no examination provider's instructions are available. Under these conditions blood cellular RNA profile changes can still occur (Figure B.1).		

6.3 Isolation of the cellular RNA

6.3.1 General

To avoid a cross contamination with amplified material, the isolation of the cellular RNA should not be performed in the same area as the amplification steps of the examination process, unless a closed system is used, which is designed to avoid cross-contamination.

6.3.2 Using blood collection tubes with RNA profile stabilizers

When processing blood from tubes containing blood cellular RNA profile stabilizers, kits specified by the manufacturer of the blood collection tube should be used for the isolation of cellular RNA. The blood collection tube and kit manufacturer's instructions for isolating the cellular RNA shall be followed.

If the specifications of the examination provider require the use of a dedicated commercially available kit, then this shall be used instead in accordance with the instructions of the examination provider.

Alternative isolation procedures can be used, if no examination provider's instructions are available and if they are verified for the same requirements and validated for the same intended use. In this case, the instructions for the validated alternative for isolating the cellular RNA shall be followed.

NOTE 1 When using alternative isolation procedures, dedicated measures and technologies can be needed in order to avoid carrying over cellular RNA stabilization molecules to the final RNA eluate. Stabilization molecules carry-over can lead to an inhibition of the examination reaction.

NOTE 2 Dedicated procedures for processing frozen specimens/samples can be included in the RNA isolation kit manufacturer's or examination provider's instructions.

6.3.3 Using blood collection tubes without RNA profile stabilizers

6.3.3.1 When using blood collection tubes not containing any blood cellular RNA profile stabilizers, the examination provider's instructions for cellular RNA isolation shall be followed.

6.3.3.2 When using blood collection tubes not containing any blood cellular RNA profile stabilizers, where there are no examination provider's instructions available, a commercially available kit for the isolation of blood cellular RNA should be used. The kit manufacturer's instructions for isolating the cellular RNA shall be followed.

Alternative isolation procedures can also be used if they are verified for the same requirements and validated for the same intended use. In this case, the instructions for this validated alternative for isolating the cellular RNA shall be followed.

The laboratory's development, verification and validation of this alternative blood cellular RNA isolation procedure, shall specifically include the verification and validation of the entire cellular RNA isolation process including the compatibility of the reagents and substances used. The laboratory shall also verify if it is necessary to transfer the blood specimen into a lysis buffer (e.g. guanidine isothiocyanate based solution) without delay after specimen reception to avoid changes in the RNA profile of interest.

A DNase treatment step should be incorporated into the cellular RNA isolation procedure where the examination is sensitive to DNA contamination in the RNA eluate^{[22][23]}.

The DNase, other reagents and consumables coming in contact with the cellular RNA shall be RNase-free.

The extracted RNA should be kept at 2 °C to 8 °C (e.g. cooling block) or on wet ice and should be examined without delay. For longer storage and long-term storage see 6.5.

6.3.3.3 If blood collection tubes without any blood cellular RNA profile stabilizers are used and these contain frozen specimens, thawing should be avoided as this can lead to immediate degradation of cellular RNA. To limit cellular RNA degradation, the still frozen blood should be transferred from the blood collection tube directly into a blood cellular RNA profile stabilizing extraction buffer and immediately homogenized.

For the isolation of cellular RNA from the homogenized sample, a verified and validated protocol shall be used.

The extracted RNA should be kept at 2 °C to 8 °C (e.g. cooling block) or on wet ice and should be assayed without delay. For longer storage and long-term storage, see 6.5.

6.4 Quantity and quality assessment of isolated cellular RNA

The RNA quantity and quality should be checked according to the examination provider's instructions or according to validated procedures by generally accepted physical, chemical and biochemical procedures^{[18][24][25][26]}. These may include one or more of the following:

- a) quantification by absorbance measurements (A_{260}) or spectrofluorometry;
- b) test for purity by absorbance measurements (e.g. wavelength scan, A_{260}/A_{280} ratio, A_{260}/A_{230} ratio);
 EXAMPLE Pure RNA has an A_{260}/A_{280} ratio of 1,9 to 2,1 in 10 mM Tris·Cl pH 7,5 (this can also be written as 10 mM Tris·HCl pH 7,5).
- c) test for RNA integrity (by e.g. electrophoresis, chromatography, molecular methods such as the 3'/5' assay or differential length amplicon ratio^{[26][27]}, or electrophoretic methods to determine the RNA integrity number (RIN) or equivalent);
- d) test for presence of interfering substances [using exogenous controls (spiked in RNA and DNA controls) or inspecting qPCR response curves for anomalies^[28] or using an endogenous RNA for an RT-PCR inhibition test by introducing increasing eluate volumes into the examination].

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

6.5 Storage of isolated cellular RNA

6.5.1 General

For long-term storage, the isolated cellular RNA is usually frozen. However, for cellular RNA preservation, other validated methods for archiving can also be used, see, for example, Reference [29].

For long-term storage, aliquots of the isolated cellular RNA should be generated to avoid repeated freezing and thawing or repeated recovery from other archiving systems.

For small RNA amounts, storage vessels with reduced nucleic acid adsorption to the tube wall should be used.

Unintended freeze-drying of the isolated cellular RNA during long-term storage due to water evaporation should be avoided as the RNA can degrade and the recovery from the tube can be difficult or even impossible. Therefore, appropriate storage vessels, such as screw-capped cryogenic vials, avoiding water evaporation during long-term storage, should be used and documented.

Traceability shall be ensured. For long-term storage, a validated system to organize and uniquely mark aliquots in the intended storage temperature making them easily retrievable and identifiable should be in place. Labels suitable for storage temperature with readable 1D- or 2D-barcodes or pre-printed tubes with unique codes provided by manufacturers are recommended to avoid loss or confusion of sample identity.

6.5.2 Cellular RNA isolated with commercially available kits

For storing the isolated cellular RNA before the examination, the RNA isolation kit provider's specific instructions should be followed. Where the examination provider's instructions are more stringent, these shall be followed.

6.5.3 Cellular RNA isolated with the laboratory's own protocols

If the laboratory's own validated cellular RNA isolation procedures are used, the isolated cellular RNA should be assayed without delay. Otherwise, the laboratory shall have verified procedures in place on how to store the isolated cellular RNA before examination.

Depending on the RNA isolation procedure and the resulting RNA eluate quality, storage on wet ice for a short period of time (i.e. 30 min) can be appropriate in certain circumstances.

For long-term storage, isolated cellular RNA should be eluted in an appropriate buffer and stored at $\leq -70\text{ }^{\circ}\text{C}$ [21]. Other validated methods for archiving can also be used, see, for example, Reference [29].

NOTE Some cellular RNA isolation procedures can allow long-term storage of the isolated cellular RNA in the range from $-20\text{ }^{\circ}\text{C}$ to $-70\text{ }^{\circ}\text{C}$.

Annex A (informative)

Impact of pre-examination process steps on venous whole blood cellular RNA profiles

A.1 General information about operated experiments in [Annex A](#) and [Annex B](#)

The Standardization and Improvement of Generic Pre-analytical Tools and Procedures for In Vitro Diagnostics (SPIDIA¹⁾) was a European Commission funded four-and-a-half-year project aiming at the standardization and improvement of pre-examination procedures for in vitro diagnostics[30].

The SPIDIA consortium performed two consecutive pan-European ring trials for identifying and improving critical pre-examination process steps influencing venous whole blood cellular RNA profile examination[13][24]. A total number of 93 and 109 European laboratories participated in ring trial 1 and 2, respectively. The second ring trial protocols included improvements worked out by the SPIDIA consortium. Two proficiency blood specimens from single donors, both either with a blood cellular RNA profile stabilizer (PAXgene²⁾ Blood RNA tubes) or without (K₂EDTA tubes), were prepared at a central SPIDIA laboratory and subsequently shipped to the participating laboratories. The specimens were shipped in dedicated boxes in order to maintain a constant temperature range of 2 °C to 8 °C.

The participants were asked to extract the cellular RNA from one tube immediately after receipt (within 24 h after blood collection) and from the second tube 24 h later (within 48 h after blood collection) after storage at either ambient or refrigerated temperature. The participants were asked to isolate the cellular RNA using their routine laboratory procedure and to send the purified RNA samples back on dry ice to the SPIDIA laboratories. At the SPIDIA laboratories, the RNA samples were evaluated for purity, yield, integrity, and presence of interfering substances. In order to analyse the impact of pre-examination variables on the blood cellular RNA profiles, the amounts of selected transcripts in the RNA samples were determined by validated marker analytical tests[14]. These included RT-qPCR assays for FBJ murine osteosarcoma viral oncogene homologue (FOS), Interleukin 8 (IL8), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), FBJ murine osteosarcoma viral oncogene homologue B (FOSB) and tumour necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain (TNFRSF10C).

Results presented below show important findings.

A.2 Influence of blood collection tube type (with or without blood cellular RNA profile stabilizer) on the analysis of specific blood cellular RNA profiles

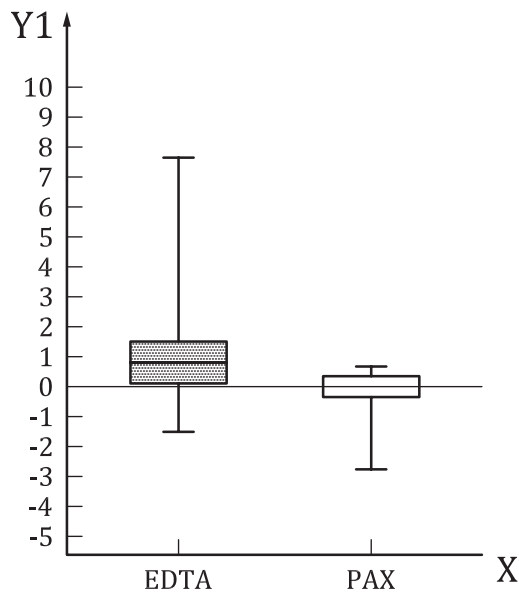
A.2.1 Unstable blood cellular RNA profiles

[Figure A.1](#) shows the unstable expressions of four genes in blood during the pre-examination process.

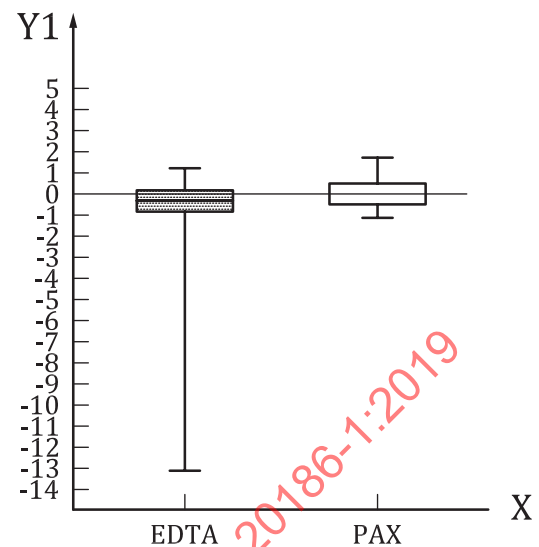
Specimens were collected either in blood collection tubes without a blood cellular RNA profile stabilizer (K₂EDTA tube, EDTA, $n = 78$) or with a blood cellular RNA profile stabilizer (PAXgene Blood RNA tube, PAX, $n = 28$). The specimens were shipped at 2 °C to 8 °C to the participating laboratories and the cellular RNA was isolated within 24 h after blood collection[13].

1) Results incorporated in this standard received funding from the European Union's Seventh Framework Programme under grant agreement No 222916. For further information see www.spidia.eu.

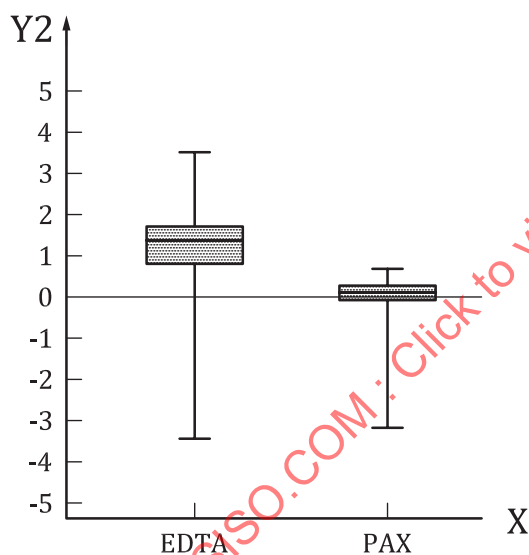
2) PAXgene is the trade name of a product supplied by PreAnalytiX GmbH. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.



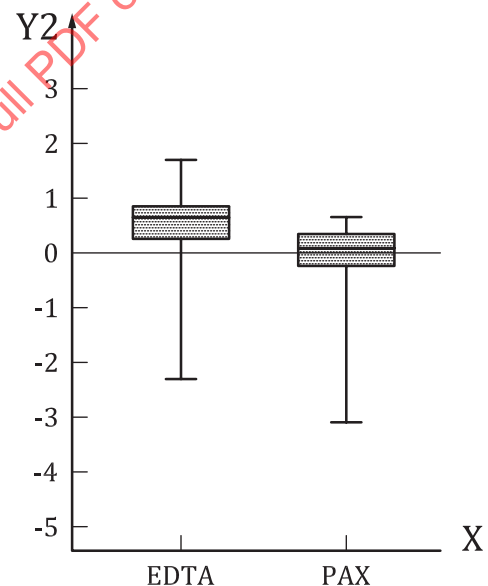
a) FOSB mRNA



b) TNFRSF10C mRNA



c) IL8 mRNA



d) FOS mRNA

Key

X blood collection tube type: EDTA, PAX

Y1 $\log_2(RQ)$

Y2 sample value – T_0 value = $\left[\log_{10} (\text{copies} / \mu\text{g isolated RNA}) \right]_{\text{sample}} - \left[\log_{10} (\text{copies} / \mu\text{g isolated RNA}) \right]_{T_0}$

NOTE 1 Boxes show the upper and lower quartile ranges (25th centile to 75th centile). The upper and lower whiskers show the maximum and minimum individual values and the line inside each box shows the median value per data set. The horizontal base-line in each graph represents the measurement at time 0 (T_0 , immediate RNA isolation after blood collection)

NOTE 2 The quantification of FOSB mRNA in a) and TNFRSF10C mRNA in b) was performed by Relative Quantification (RQ) using T_0 as calibrator, and normalized to the two housekeeping genes [Peptidyl-Prolyl cis-trans Isomerase B (PPIB) or cyclophilin B and β -Glucuronidase (GUSB)]. RQ is calculated with:

$$RQ = 2^{-\Delta\Delta Cq}$$

$$\Delta\Delta Cq = \Delta Cq_{24h} - \Delta Cq_{T_0}$$

$$\Delta Cq = Cq_{\text{target}} - Cq_{\text{housekeeping genes}}$$

where Cq is the quantification cycle^[22].

NOTE 3 The quantification of IL8 mRNA in c) was performed by absolute quantification using a standard curve.

NOTE 4 The statistical analysis was performed using the Kruskal-Wallis test.

Figure A.1 — Expressions of four genes in blood collected in tubes either with (PAX) or without (EDTA) a blood cellular RNA profile stabilizer measured by RT-qPCR

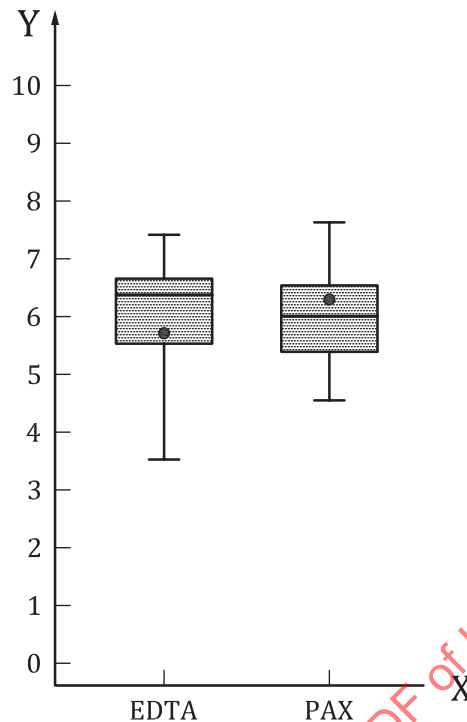
Statistically significant differences were observed between the *ex vivo* gene expressions measured in blood collected in tubes with and without blood cellular RNA profile stabilizers (p -value < 0,001 for all examined transcripts).

The results demonstrate that blood cellular RNA profile changes can still occur at 2 °C to 8 °C. Blood cellular RNA profile stabilizers in blood collection tubes are an important contributor to avoid or reduce changes in blood cellular RNA profiles during pre-examination processes and to keep them close to the native state at T_0 .

A.2.2 Stable blood cellular RNA profiles

[Figure A.2](#) shows the stable GAPDH *ex vivo* gene expression in blood collected in tubes with and without a blood cellular RNA profile stabilizer.

Specimens were collected either in blood collection tubes without a blood cellular RNA profile stabilizer (K₂EDTA tube, EDTA, $n = 78$) or with a blood cellular RNA profile stabilizer (PAXgene Blood RNA tube, PAX, $n = 28$). The specimens were shipped at 2 °C to 8 °C to the participating laboratories and the blood cellular RNA was isolated within 24 h after blood collection^[13].

**Key**

X blood collection tube type: EDTA, PAX

Y sample value = $\log_{10}$ (copies/ μg isolated RNA)

NOTE 1 Boxes show the upper and lower quartile ranges (25th centile to 75th centile). The upper and lower whiskers show the maximum and minimum individual values and the line inside each box shows the median value per data set. The dot in each box represents the measurement at time 0 (T_0 , immediate RNA isolation after blood collection)[13].

NOTE 2 The quantification of GAPDH mRNA was performed by absolute quantification using a standard curve.

NOTE 3 The statistical analysis was performed using the Kruskal-Wallis test.

Figure A.2 — Stable expression of GAPDH in blood collection tubes either with (PAX) or without (EDTA) a blood cellular RNA stabilizer measured by RT-qPCR

The analyses showed no statistically significant difference between the *ex vivo* expression of the GAPDH gene at T_0 and after 24 h in both tubes, with and without a blood cellular RNA profile stabilizer. This demonstrated that *ex vivo* expression of certain genes such as GAPDH is in some cases not affected by storage and transport (see [Figure A.2](#)).

This also demonstrates that the main process for blood cellular RNA profile changes (see [Figure A.1](#)) is altered gene expression rather than general cellular RNA degradation which would affect all transcripts, including stable transcripts.

Annex B **(informative)**

Influence of blood storage temperature on blood cellular RNA profiles

[Figure B.1](#) shows the unstable expression of three genes in blood when collected in tubes without a blood cellular RNA profile stabilizer and stored for 48 h at room temperature (RT) or 2 °C to 8 °C.

Specimens were collected either in blood collection tubes without a blood cellular RNA profile stabilizer (K₂EDTA tube, EDTA, $n = 78$) or with a blood cellular RNA profile stabilizer (PAXgene Blood RNA tube, PAX, $n = 28$). The blood specimens were shipped at 2 °C to 8 °C to the participating laboratories. The blood in the stabilizer containing tubes was stored at room temperature (PAX-RT, $n = 28$), the blood collected in tubes without a blood cellular RNA profile stabilizer was stored at 2 °C to 8 °C (EDTA-4 °C, $n = 39$) or at room temperature (EDTA-RT, $n = 39$) at the participating laboratories. Cellular RNA was isolated 48 h after blood collection^[13].

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