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BSI Standards Publication

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

Part 1: Isolated cellular RNA

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Tests de diagnostic moléculaire in vitro - Spécifications relatives aux processus préanalytiques pour le sang veineux total - Partie 1 : ARN cellulaire isolé

Molekularanalytische in-vitro-diagnostische Verfahren - Spezifikationen für präanalytische Prozesse für venöse Vollblutproben - Teil 1: Isolierte zelluläre RNS

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Contents

Page

Foreword

This document (CEN/TS 16835-1:2015) 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 analyzing signatures of nucleic acids, proteins, and metabolites in human tissues and body fluids. However, the profiles 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 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 venous whole blood cellular RNA analysis in what is referred to as the preanalytical phase.

1 Scope

This Technical Specification recommends the handling, documentation and processing of venous whole blood specimens intended for cellular RNA analysis during the preanalytical phase before a molecular assay is performed. This Technical Specification covers specimens collected by venous whole blood collection tubes. This Technical Specification is applicable to molecular *in vitro* diagnostic examinations (e.g. *in vitro* diagnostic laboratories, laboratory customers, *in vitro* diagnostics developers and manufacturers, institutions and commercial organizations performing biomedical research, biobanks, and regulatory authorities).

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

Different dedicated measures need to be taken for stabilizing blood cell free circulating RNA and RNA in exosomes circulating in blood, which are not described in this Technical Specification.

Different dedicated measures need to be taken for collecting, stabilizing, transporting and storing capillary blood as well as for collecting and storing blood by paper based technologies. These are not described in this Technical Specification.

RNA in pathogens present in blood is not covered by this Technical Specification.

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 kind of parameter testing or chemical manipulation for quantitative or qualitative analysis

3.3
blood cellular RNA
cellular RNA
RNA molecules present in blood cells

3.4
blood cellular RNA profile\s
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

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

3.6
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), temporary storage, transportation to and within the analytical laboratory, aliquotting, retrieval, isolation of analytes, and end when the analytical examination begins

[SOURCE: EN ISO 15189:2012, 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.

3.7
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.]

3.8
RNA
ribonucleic acid
polymer of ribonucleotides occurring in a double-stranded or single-stranded form

[SOURCE: EN ISO 22174:2005, 3.1.3]

3.9
room temperature
temperature which is defined as 18 °C to 25 °C for the purposes of this document

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 blood cellular RNA.

4 General considerations

For general statements on primary sample collection and handling (including avoidance of cross contaminations), see EN ISO 15189:2012, 5.2.6, 5.4.4. 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 sample stability and storage conditions, and the analytical steps should be verified and validated (see EN ISO 15189).

Blood cellular RNA profiles can change significantly after collection (e.g. gene induction, gene down regulation, RNA degradation) [3], [4], [5], [6]. These changes can vary individually in different blood donors' / patients' blood [3], [7], [8], [9], [10].

The stability of the specific blood cellular RNA profile of interest should be investigated throughout the complete preanalytical workflow.

Before or during the design of the analytical test system it should be investigated and ensured that the specific blood cellular RNA molecule/s amount/s intended to be analyzed in the analytical test is/are not affected by the envisioned entire preanalytical workflow.

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.

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

5 Outside the laboratory

5.1 Primary venous whole blood 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 and relevant lifestyle factors of the blood donor (e.g. healthy, disease type, diet, gender, age);

c) the information about medical treatment and special treatment prior to blood collection (e.g. anaesthetics, medications, fasting status);

d) the type and purpose of the analytical test requested.

See also EN ISO 15189:2012, 5.4.4.

5.1.2 Selection of the venous blood collection tube by the laboratory

Due to the high instability of blood cellular RNA profiles in individual patients/donors [3], [7], [8], [9], [10], commercially available blood collection tubes containing blood cellular RNA profile stabilizers should be used [7], [8], [10], [11], [12] (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 analyzed is stable after blood draw (Figure A.2) or if the requested analytical test allows the use of such tubes.

5.1.3 Primary venous whole blood collection from the patient and stabilization procedures

1. The identity of the person collecting the sample and the time of blood collection according to EN ISO 15189:2012, 5.4.4.3, f) shall be documented.

2. For the labelling (sample identification) of the blood collection tube a routine procedure (EN ISO 15189:2012, 5.4.4.3, e)) or a procedure with additional information (e.g. 2D-barcode) shall be used.

3. Standard venepuncture technique can be used. Steps for preventing possible backflow may be required. The manufacturers' 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 There is no known specific effect of venous whole blood draw procedure on the cellular RNA. Routine procedures can therefore be used.

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

5. Blood collection tube manufacturers' instructions, for mixing or inverting the tube immediately after blood collection, shall be followed.

   NOTE Unless additives are homogenously mixed with the blood sample, the blood cellular RNA profile quality and the quality of individual cellular RNA molecules can be compromised, which can impact the validity and reliability of the analytical test results.

6. Any tampering with and/or additions to the primary sample shall be documented.

5.1.4 Information on the primary blood sample and storage requirements at the blood collection facility

The documentation on the primary blood sample shall include the date and time of blood collection [3], [14], [15], [16] as blood cellular RNA profiles can change significantly after blood collection and can thereby affect the validity and reliability of the analytical test result [3], [11], [13],[14].

For storing the primary blood samples collected in blood collection tubes with blood cellular RNA profile stabilizers, the blood collection tube manufacturers' instructions on storage conditions shall be followed (temperature and storage duration). Where the analytical test providers' instructions are more stringent, these shall be followed. The storage conditions (storage duration and temperature) shall be documented.

Blood collection tubes without blood cellular RNA profile stabilizers should only be used, if the ordered analytical test specifications allow the usage of such tubes. In these cases, the analytical test providers' instructions on storage conditions shall be followed. This can require documentation of storage conditions.

When using blood collection tubes without blood cellular RNA profile stabilizers and no requirements on the storage conditions are available, the primary blood samples should be transferred immediately to 2 °C to 8 °C or on wet-ice in order to minimize blood cellular RNA profile changes [8], [14], [15], [16] (Figure B.1). The storage conditions (storage duration and temperature) shall be documented. The storage duration allowed at 2 °C to 8 °C or on wet-ice is highly dependent on the stability of the individual RNA molecules and their cellular quantities to be analyzed in the analytical test. This stability can vary between several minutes and over 24 h.

NOTE Under these storage conditions (at 2 °C to 8 °C or on wet-ice) blood cellular RNA profile changes can still occur when no blood cellular RNA profile stabilizers are used (Figure B.1).

For samples dedicated to be archived in a biobank it is usually not known which individual RNA molecules will be analyzed after archiving, therefore tubes without blood cellular RNA profile stabilizers should not be used for biobanking.

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

5.2 Transport requirements

The required transport conditions shall be documented including any deviations.

When using blood collection tubes with blood cellular RNA profile stabilizers, the tubes' manufacturers' instructions on transport conditions shall be followed (e.g. temperature, transport duration). Where the analytical test providers' instructions are more stringent, these shall be followed.

When using blood collection tubes without blood cellular RNA profile stabilizers, the analytical test providers´ instructions on transport conditions shall be followed. This can require the documentation of transport conditions (duration and temperature).

When using blood collection tubes without blood cellular RNA profile stabilizers and no analytical test provider's instructions are available, the primary blood sample 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 [16].

See also EN 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 Sample reception

The blood sample reception time shall be documented. Nonconformities of labelling, transport conditions and blood volume differences to specifications, leaking/broken tubes, etc. shall be documented.

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 analytical test result, a new sample should be obtained.

6.2 Storage requirements

The storage temperature and time interval between sample 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 primary blood sample total storage duration shall include the duration for storage at the blood collection facility (5.1.4), for transportation to the laboratory (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 (e.g. mRNA, rRNA, miRNA) different blood donors, and different blood storage conditions. Any specified maximum storage duration given by the blood collection tube manufacturer or the analytical test manufacturer shall not be exceeded. If such specifications are not available, the maximum storage duration shall be validated 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 stabilizers

Blood collection tube	Blood collection, transport and storage	
	Duration	Temperature
With blood cellular RNA profile stabilizer	— Blood collection tube manufacturer's instructions — Analytical test providers' instructions[a]	
Without blood cellular RNA profile stabilizer	— Analytical test instructions	— Analytical test instructions — Wet-ice or 2 °C to 8°C[b]

[a] If more stringent than blood tube manufacturer's instructions.

[b] Under these conditions blood cellular RNA profile changes can still occur (Figure B.1).

6.3 Isolation of the cellular RNA

The laboratory shall validate the entire process comprising sample collection, storage, transportation and isolation of cellular RNA to the final analytical test result according to its internal quality management system (see EN ISO 15189).

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

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. Alternative extraction procedures can be used if verified for the same requirements and validated for the same intended use.

NOTE 1 When using alternative extraction procedures, dedicated measures and technologies might be needed in order to avoid carrying over cellular RNA stabilization molecules to the final RNA eluate. Carry-over from stabilizer molecules can lead to an inhibition of the analytical test reaction.

Blood collection tube and kit manufacturers' instructions or the instructions for the validated alternative for isolating the cellular RNA shall be followed.

NOTE 2 Dedicated procedures for use for processing frozen blood samples can be included in the manufacturers' instructions.

When using blood collection tubes not containing any blood cellular RNA profile stabilizer, the analytical test manufacturers' instructions or validated alternatives for cellular RNA isolation shall be followed.

When using blood collection tubes not containing any blood cellular RNA profile stabilizer, where there are no analytical test manufacturers' instructions, the laboratory shall validate the entire cellular RNA isolation process.

A DNase treatment step should be incorporated into the cellular RNA isolation procedure [17], [18]. The DNase, other reagents and consumables coming in contact with the cellular RNA sample should be RNase-free.

If blood collection tubes containing frozen samples without any blood cellular RNA profile stabilizer are used, thawing should be avoided as this can lead to immediate degradation of cellular RNA. To limit cellular RNA degradation, the frozen blood should be transferred from the blood collection tube directly into a blood cellular RNA profile stabilizing extraction buffer and immediately be homogenized. The homogenized sample should be further cleaned up and concentrated by validated protocol steps. The extracted RNA should be kept at

2 °C to 8 °C (e.g. cooling block) or on wet-ice and should be assayed immediately. For longer storage and archiving see 6.5.

6.4 Quality assessment of isolated cellular 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 [13], [19], [20]. 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[21]);

d) test for presence of interfering substances (using exogenous controls (spiked in RNA and DNA controls) or inspecting qPCR response curves for anomalies [22]).

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

6.5 Storage of isolated cellular RNA

For storing and archiving the isolated cellular RNA (before the analytical phase), the RNA isolation kit providers' specific instructions should be followed.

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

Storage on wet-ice for a short period of time (i.e. 30 min) can be appropriate in certain circumstances.

Storage for archiving purposes should be at −70 °C or below [16].

Some cellular RNA isolation procedures may allow archiving the isolated cellular RNA at −20 °C to −70 °C.

For archiving, aliquots of the isolated cellular RNA should be generated to avoid freezing and thawing.

Unintended freeze-drying of the isolated cellular RNA during archiving 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 tubes avoiding water evaporation during archiving should be used.

Annex A
(informative)

Impact of preanalytical workflow steps on venous whole blood cellular RNA profiles

A.1 General information on operated experiments in Annex A and Annex B

SPIDIA[1)] (Standardisation and Improvement of Generic Pre-analytical Tools and Procedures for In Vitro Diagnostics; www.spidia.eu) was a European Commission funded four-and-a-half-year project aiming at the standardization and improvement of preanalytical procedures for *in vitro* diagnostics [23]).

The SPIDIA consortium performed two consecutive pan-European ring trials for identifying and improving critical preanalytical workflow steps influencing venous whole blood cellular RNA profile analysis [8], [19]. 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 (PAXgene[2)] Blood RNA tubes) or without (K_2EDTA tubes), were prepared at a central SPIDIA laboratory and subsequently shipped to the participating laboratories. The samples 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 analyze the impact of preanalytical variables on the blood cellular RNA profiles, the amounts of selected transcripts in the RNA samples were determined by validated marker assays [9]. These included RT-qPCR assays for FOS (FBJ murine osteosarcoma viral oncogene homologue), IL8 (Interleukin 8), GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), FOSB (FBJ murine osteosarcoma viral oncogene homologue B) and TNFRSF10C (tumour necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain).

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 preanalytical workflow.

Blood samples were collected either in blood collection tubes without a blood cellular RNA profile stabilizer (K_2EDTA tube, EDTA, n = 78) or with a blood cellular RNA profile stabilizer (PAXgene Blood RNA tube, PAX,

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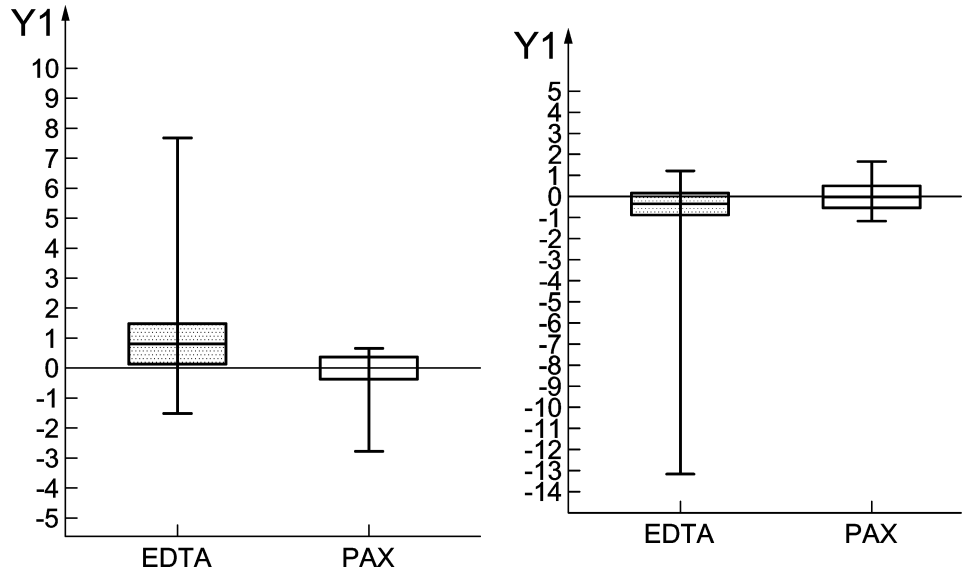
1) Research funded by the European Union Seventh Framework Programme [FP7/2007-2013] under grant agreement no 222916.

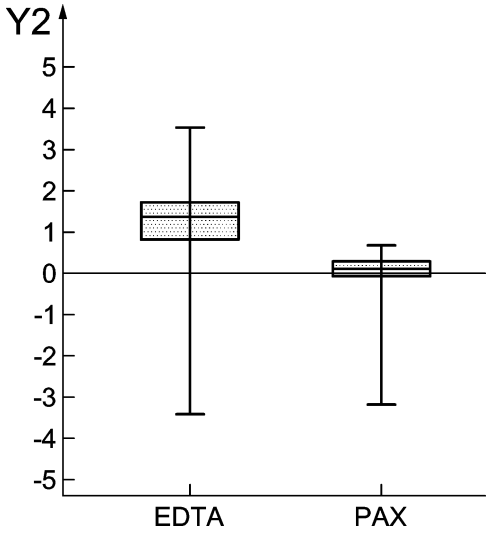
2) PAXgene is the trade name of a product supplied by PreAnalytiX GmbH. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by CEN or CENELEC of the product named. Equivalent products may be used if they can be shown to lead to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

n = 28). The blood samples were shipped at 2 °C to 8 °C to the participating laboratories and the cellular RNA was isolated within 24 h after blood collection [8].

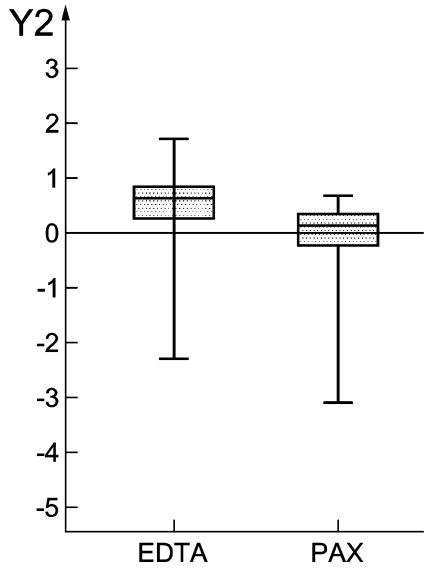


a) FOSB mRNA

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 = $(\log_{10}(\text{copies/µg total RNA}))_{\text{sample}} - (\log_{10}(\text{copies/µg total RNA}))_{T_0}$

NOTE 1 Boxes show the upper and lower quartile ranges (25^{th} centile to 75^{th} 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) [8].

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 (PPIB: Peptidyl-Prolyl cis-trans Isomerase B or cyclophillin B; GUSB: β-Glucuronidase). RQ is calculated with:

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

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

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

where

Cq is the quantification cycle [17].

NOTE 3 The quantification of IL8 mRNA in c) and FOS mRNA in d) 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

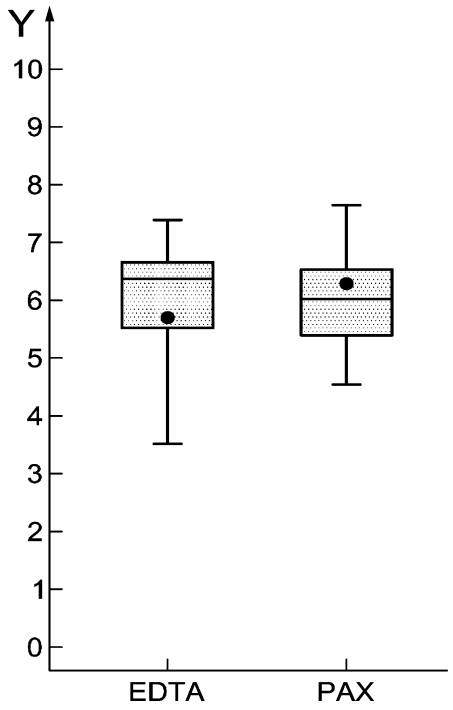
Statistically significant differences were observed between the *ex-vivo* gene expressions measured in blood collected in tubes with and without blood cellular RNA profile stabilizers (p-value < 0,001 for all analyzed transcripts).

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 preanalytical workflows 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.

Blood samples were collected either in blood collection tubes without a blood cellular RNA profile stabilizer (K_2EDTA tube, EDTA, n = 78) or with a blood cellular RNA profile stabilizer (PAXgene Blood RNA tube, PAX, n = 28). The blood samples 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 [8].



Key

X blood collection tube type: EDTA, PAX Y sample value = $\log_{10}$ (copies/µg total RNA)

NOTE 1 Boxes show the upper and lower quartile ranges (25^{th} centile to 75^{th} 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) [8].

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 may not be affected by storage and transport (see Figure A.2).

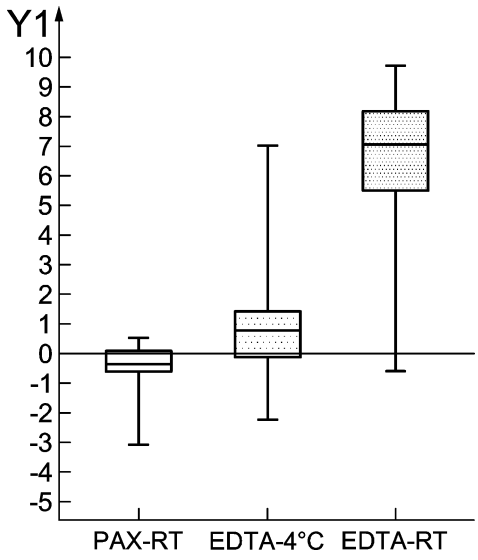
This also demonstrates that the main process for blood cellular RNA profile changes (see Figure A.1) is altered gene expression rather than general cellular RNA degradation which would affect all transcripts, including stable transcripts.

Annex B
(informative)

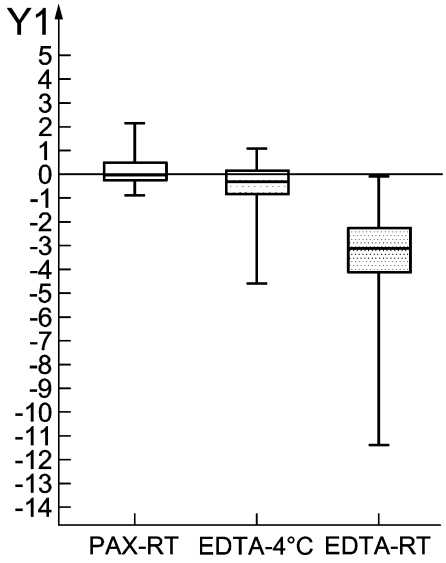
Influence of blood storage temperature on blood cellular RNA profiles

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.

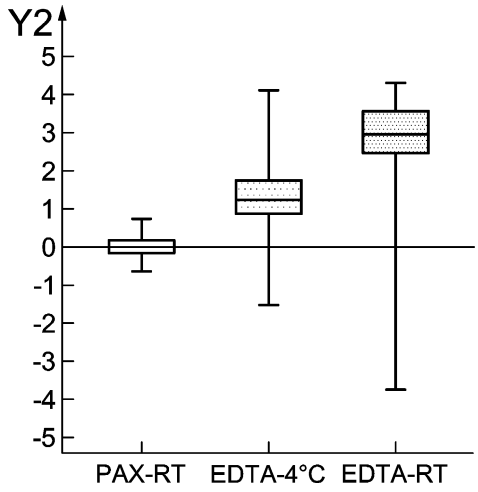
Blood samples were collected either in blood collection tubes without a blood cellular RNA profile stabilizer (K_2EDTA tube, EDTA, n = 78) or with a blood cellular RNA profile stabilizer (PAXgene Blood RNA tube, PAX, n = 28). The blood samples were shipped at 2 °C to 8 °C to the participating laboratories. 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 [8].



a) FOSB mRNA



b) TNFRSF10C mRNA



c) IL8 mRNA

Key

X blood collection tube type and storage temperature: PAX-RT, EDTA-4°C, EDTA-RT

Y1 $\log_2(RQ)$

Y2 sample value – T_0 value = $(\log_{10}$ (copies/µg total RNA)$)_{sample} - (\log_{10}$ (copies/µg total RNA)$)_{T0}$

NOTE 1 Boxes show the upper and lower quartile ranges (25^{th} centile to 75^{th} 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) [8].

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 (PPIB: Peptidyl -prolyl cis-trans isomerase B or cyclophillin B; GUSB: β-glucuronidase). 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 [17].

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 B.1 — Expression of three genes in blood collected in tubes either with (PAX) or without (EDTA) a blood cellular RNA profile stabilizer at different temperatures measured by RT-qPCR

Statistically significant differences were observed between the FOSB, TNFRSF10C and IL8 *ex-vivo* gene expression changes measured in tubes without a blood cellular RNA profile stabilizer stored at 2 °C to 8 °C and at room temperature (p-value < 0,001 for all analyzed transcripts). However, *ex-vivo* gene expression changes, compared to the native state at T_0, were lower for blood samples stored at 2 °C to 8 °C.

These gene expressions differed statistically significant also from the gene expressions measured for blood stored in tubes containing a blood cellular RNA profile stabilizer, which kept the gene expressions close to the native state at T_0.

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