Impact of RNA Stabilizing Blood Collection Tubes on Gene Expression Data Validity – A Comparison of S-Monovette® RNA Exact, PAXgene™ Blood RNA Tubes & Tempus™ Blood RNA Tubes

Linden J.[1], Kämper M.[1], Schulz D.[2], Eikelmann G.[1] & Schuster R.[1,2]

[1] SARSTEDT AG & Co. KG, Research & Development, Nümbrecht, Germany
[2] SARSTEDT AG & Co. KG, Marketing & Product Management, Nümbrecht, Germany

Abstract

Gene expression analysis based on RNA isolated from whole blood is an important diagnostic method but bears several pre-analytical hurdles, e. g. instability of transcripts and unintended gene induction *ex vivo*. Throughout the past decade, a variety of blood collection tubes have been launched to address these issues. However, the currently available blood collection devices have certain limitations in RNA stabilization and/or in the appropriate isolation procedures.
Here we introduce the new SARSTEDT S-Monovette® RNA Exact. Whole blood can be stored within the tube for at least 5 days at room temperature, at least 14 days at 8 °C and at least 36 months at -80 °C while maintaining RNA amount and integrity. Moreover, we tested the expression levels of five genes that have been suggested by the SPIDIA consortium to monitor pre-analytical gene expression changes [1]. All five genes are known to exhibit *ex vivo* induced expression level changes and were shown to be efficiently stabilized by the S-Monovette® RNA Exact for the periods indicated above.
All in all, the S-Monovette® RNA Exact proves to stabilize RNA levels more efficiently than two currently established RNA stabilizing blood collection systems and can serve as a highly performant device for blood collection, transport, storage and isolation.

Introduction

Gene transcription analysis based on the expression level of ribonucleic acid (RNA) is a common tool to study the functional activity of cells. Gene expression information are of important use in research and in clinical practice, e.g. to diagnose and monitor diseases [2, 3], to predict genetic risks of diseases [4] or to follow up the effects of therapeutic interventions [5]. Due to the minimal invasive character of sample collection, blood based diagnostics allow easy, rapid and convenient determination of the individuals' health state and are the gold standard in routine diagnostics.
For clinical diagnostics based on RNA from whole blood, there are critical points to be considered even prior to the analysis of the sample, namely blood collection (1), transport (2) and storage (3) until further processing. Several studies have shown a big impact of the choice of the respective blood collection tube and the storage conditions on RNA-based molecular diagnostics. For example, it has been known for several years that the usual anticoagulating additives of blood collection tubes, e. g. EDTA, are not able to conserve gene expression levels in blood samples after phlebotomy [6, 7]. RNA levels are affected by two major factors: (1) RNA is rapidly degraded *ex vivo* by nucleases or heat [8, 9] and (2) gene transcription might be dysregulated by various causes after phlebotomy, e.g. during sample collection, sample storage (blood coagulation) or hemolysis (exposure of free RNA to cell contents such as hemoglobin) [10]. This dysregulation is detectable shortly after blood collection and renders the sample useless for gene expression analysis and subsequent diagnostics.
Within the SPIDIA project (Standardization and improvement of generic pre-analytical tools and procedures for *in vitro* diagnostics), funded by the European Commission, the need for quality standards and improvement of pre-analytical procedures for RNA-based *in vitro* diagnostics have been pointed out [11, 12]. One major conclusion from the SPIDIA project was that gene expression analysis is only possible by stabilization of RNA right after phlebotomy [12].
For this purpose, several manufacturers have launched pre-evacuated blood collection tubes containing additives which lyse blood cells immediately during/after blood collection. These blood collection tubes are intended to stabilize the RNA up to several days at ambient temperatures [13].
The currently most commonly used RNA-stabilizing blood collection tubes are the PAXgene™ Blood RNA Tubes from PreAnalytix [14–16] and the Tempus™ Blood RNA Tubes from Applied Biosystems [17, 18]. Despite pronounced differences in their capability to stabilize RNA, both systems require individual centrifugation steps which complex and decelerate the isolation process. A centrifugation step impedes an easily automated process and elongates the RNA isolation process considerably.
Both systems have preferentially been used for storage of frozen blood [19, 17, 20]. It should be noted that both systems show aberrant expression levels of certain

genes and thus do not stabilize all mRNAs equally [21, 22]. Since gene transcription-based diagnostics gain more and more importance [3, 23, 24] there is a clear and present need to improve RNA stabilization and adapt this technology for routine diagnostics.
Here we describe the quick and easy workflow of RNA isolation from blood samples stabilized by the new SARSTEDT S-Monovette® RNA Exact blood collection tube. We compare the quality of isolated RNA to those isolated from blood stabilized by PAXgene™ and Tempus™ blood collection tubes. We demonstrate that the S-Monovette® RNA Exact preserves cellular RNA in whole blood for at least 6 days at room temperature and 14 days at 8 °C as well as at least 36 months at -80 °C (study ongoing).

Materials & Methods

Blood collection & storage

Blood from 8 healthy donors was drawn into S-Monovette® RNA Exact blood collection tubes (Cat. No.: 01.2048.001, SARSTEDT AG & Co. KG, Nümbrecht, Germany), Tempus™ Blood RNA tubes (Applied Biosystems, Foster City, CA, USA) and PAXgene™ Blood RNA tubes (PreAnalytiX GmbH, Hombrechtikon, Switzerland). Samples were incubated at different temperatures. After incubation, stabilized whole blood was frozen at -80 °C until RNA isolation.

RNA isolation

The PAXgene™ tubes contain 2.5 ml blood from which RNA was isolated with the recommended manual kit (PAXgene® Blood RNA Kit 50,v2, #762174, PreAnalytiX GmbH) according to manufacturer's instructions and eluted into a total volume of 100 µl (two times 50 µl, protocol indicates two times 40 µl). Tempus™ tubes contain 3.0 ml blood from which RNA was isolated according to manufacturer's instructions with the Tempus™ Spin RNA Isolation Reagent Kit, #4378926, Applied Bioystems and eluted into 100 µl (two times flow through of the same fluid as indicated in the protocol). 2.5 ml EDTA anticoagulated blood (Cat. No.: 04.1917.001, SARSTEDT AG & Co. KG, Nümbrecht, Germany) was used as control. RNA of 2.5 ml EDTA anticoagulated blood was isolated with the Macherey-Nagel NucleoSpin® RNA Blood Midi Kit, #740210.20 (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions except that the volumes of lysis-buffer and ethanol were adapted to the higher sample volume (the manual describes isolation from 1.3 ml whole blood). The total elution volume was 200 µl. Preliminary experiments showed that the binding capacity of the midi columns was not exceeded with the use of 2.5 ml blood.
Stored whole blood samples in S-Monovette® RNA Exact were digested with proteinase K in the blood collection tubes and after, the samples were directly put onto isolation columns from the NucleoSpin® RNA Blood kit without addition of binding/lysis buffer. Due to the elevated sample volume, the column loading was performed two times. After these loading steps, RNA was isolated according to manufacturer's instructions. Eluates were stored at -80 °C until further processing as described below. An overview of this simplified isolation protocol is illustrated in Fig. 1 and a detailed description is given in the user manual of the NucleoSpin® RNA Blood Midi Kit.

RNA quantity & purity

RNA quantity was determined by measuring absorbance at 260 nm and the quality of eluates was estimated by determining the 260 nm/280 nm ratio with a BioPhotometer plus (Eppendorf, Hamburg, Germany) according to manufacturers' protocol.

RNA integrity (RIN)

RNA integrity was measured with a Bioanalyzer 2100 (Agilent, Waldbronn, Germany) using RNA Nano 6000 Chips (Agilent).

cDNA synthesis and Real-time qPCR

Purified RNA was reversely transcribed into cDNA using the First Strand cDNA Synthesis Kit (#K1612, Life Technologies, Darmstadt, Germany) according to manufacturers' instructions. RT-reactions were run only with poly(T) primers to prevent transcription of fragmented mRNA lacking a poly poly(A) tail.
To investigate RNA preservation over time, quantitative real-time PCRs of five genes were conducted. To minimize signals derived from contaminating DNA, DNase digestion was performed according to the manufacturer's instructions. Because traces of resting DNA always remain on the columns, we designed primers located directly on exon/intron boundaries or primers spanning exon/intron boundaries to prevent signals derived from remaining DNA. All primers were synthesized by Biospring GmbH (Frankfurt, Germany).

Tab. 1a: Primers used for quantitative real-time PCR

gene	accession No.	primer	amplicon length
chemokine ligand 8 (*IL-8*)	NM_000584.3	IL-8_126fw GGAAGGAACCATCTCACTGTG	151 bp
		IL-8_276rv GGAGTATGTCTTTATGCACTGAC	
interleukin 1, beta (*IL-1ß*)	NM_000576.2	IL-1B_4fw AAACCTCTTCGAGGCACAAGG	198 bp
		IL-1B_201rv GTCCTGGAAGGAGCACTTCATC	
FBJ murine osteosarcome viral oncogene homolog (*FOS*)	NM_005252.3	FOS_336fw TCAACGCGCAGGACTTCTGC	375 bp
		FOS_688rv TCTCCGCTTGGAGTGTATCAGTC	
forkhead box P3 (*FOXP3*)	NM_014009.3	FOXP3 2fw AACAGCACATTCCCAGAGTTCC	205 bp
		FOXP3 1344 rv GGATGGCGTTCTTCCAGGTGG	
tumor necrosis factor receptor superfamily member 10c (*TNFRSF10C*)	NM_003841.3	TNFRSF10C_318fw ATCCCCAAGACCCTAAAGTTCG	163 bp
		TNFRSF10C_480rv GAGATCCTGCTGGACACTCCTC	

Reference genes were chosen according to their proven expression in blood [25] and different expression levels. The commonly used housekeeping gene GAPDH was dismissed because it was not possible to prevent a non-RT-derived background signal. This signal is caused by remaining DNA of pseudogenes whose structures resemble a cDNA copy [26]. Gene expression of target genes (Cp-values) were normalized to the average expression level of three housekeeping genes [27].

Tab. 1b: Primers used for quantitative real-time PCR

gene	accession No.	primer	amplicon length
hydroxymethylbilane synthase (*HMBS*)	NM_000190.3	PBGD fw CTGGTAACGGCAATGCGGC	216 bp
		PBGD 377 rv TCTCTCCAATCTTAGAGAGTGC AG	
hypoxanthine phosohoribosyl-transferase (*HPRT1*)	NM_000194.2	HPRT1_489fw GACCAGTCAACAGGGGACAT	195 bp
		HPRT1_683rv AACACTTCGTGGGGTCCTTTTC	
guanin nucleotide binding protein (*GNB2L1*)	NM_006098.4	GNB2L1_433fw GAGTGTGGCCTTCTCCTCTG	224 bp
		GNB2L1_656rv GCTTGCAGTTAGCCAGGTTC	

All RT-qPCRs were performed with the Maxima SYBR Green/ROX qPCR Master Mix (#K0223, Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturers' protocol on a Mastercycler ep realplex 4S (Eppendorf, Hamburg, Germany). Primers were used in a final concentration of 0.5 µM. After an initial denaturation of 10 minutes at 95 °C, 40 cycles of denaturation (15 sec. at 95 °C), annealing (15 sec. at 59 °C) and elongation (15 sec. at 68 °C) were performed.

Data analysis

Median and the 25%-75% interquartile range of CT

values were calculated to present data with respect to its biological variations (inter donor variations in white blood cell content). For statistical analysis and plotting of data, Prism® was used (GraphPad Software Inc., LaJolla, CA, USA).

Results

RNA is degraded in EDTA-anticoagulated whole blood

RNA undergoes rapid turnover in human cells and is highly susceptible to degrading enzymes such as RNases. To this end, half-life of RNA in human blood is very short. To allow RNA-based diagnostics in human blood, RNA has to be stabilized right after sampling (=drawing of blood).

We analyzed the stabilizing capacity of three available RNA stabilizing blood collection tubes (PAXgene™, S-Monovette® RNA Exact and Tempus™) by determining the amount of RNA in whole blood. Samples were stored for up to six days at 22.5 °C and up to 14 days at 8 °C.

Blood was drawn into stabilizing tubes and isolated with the respective proprietary kit according to manufacturer's instructions. RNA from the S-Monovette® RNA Exact was isolated with the Macherey-Nagel NucleoSpin® RNA Blood Midi Kit. Due to the composition of the stabilization solution, the protocol was simplified (Figure 1). No additional lysis buffer and no ethanol are required to achieve binding conditions. After a proteinase K digestion, the sample is directly loaded onto the columns.

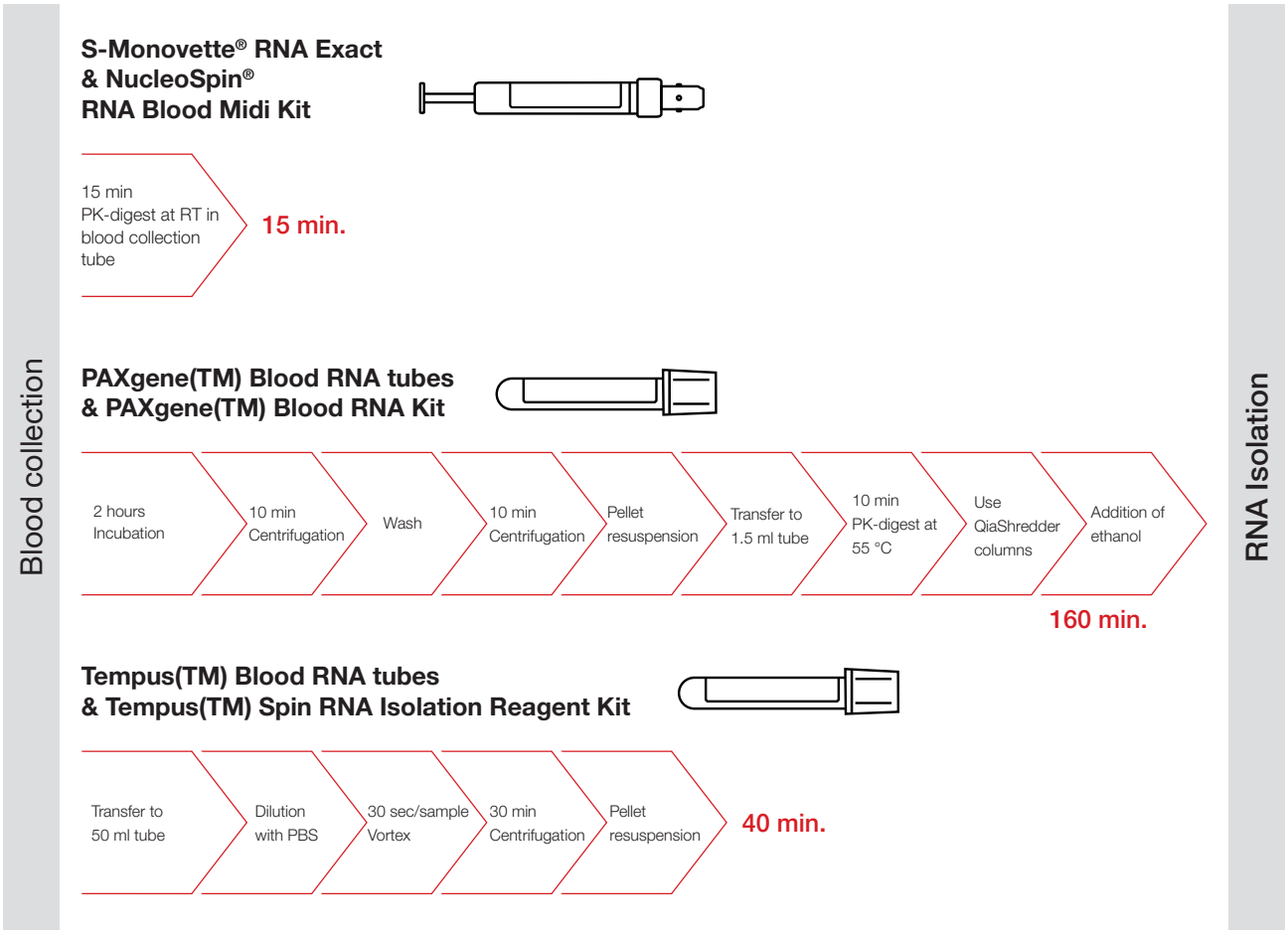

Figure 1: Workflow of three different isolation systems
Comparison of the principle workflows of RNA isolation via the manual Tempus Spin RNA Isolation system, the manual PAXgene Blood RNA system and the SARSTEDT S-Monovette® RNA Exact/ Macherey-Nagel NucleoSpin® RNA isolation system.

Photometric determination of the concentration of isolated RNA revealed that the amount of RNA received from non-stabilized (EDTA-anticoagulated) blood is not altering massively within the first day of storage. A decrease was detected only after a storage time of more than 2 days at room temperature (Fig. 2A, 2B) or after 7 days at 8 °C (Fig. 2C, 2D).

When blood was drawn into PAXgene™ tubes and RNA was isolated with the corresponding kit, the amount of RNA remained constant. However, the total yield of RNA was lower when compared to EDTA-blood. When blood was drawn into Tempus™ tubes and RNA was isolated with the corresponding kit, also lower RNA amounts were retrieved than in EDTA-blood. Astonishingly, the amount of RNA in Tempus™ tubes increased over time, reaching levels comparable to EDTA-blood after two days. When blood was drawn into the S-Monovette® RNA Exact and RNA was isolated with the Macherey-Nagel NucleoSpin® RNA Blood Midi Kit, the total amount of RNA was highest. Furthermore, the amount of RNA remained constant throughout 6 days 22.5 °C but did show some decrease throughout 14 days at 8 °C.

It is evident that the obtained RNA concentrations from the S-Monovette® RNA Exact are lower or equal in comparison to the concentrations obtained from the other blood collection devices. However, the elution volume of the NucleoSpin® RNA Blood Midi Kit is higher (see above) and thus the total amount of obtained RNA is highest from the S-Monovette® RNA Exact.

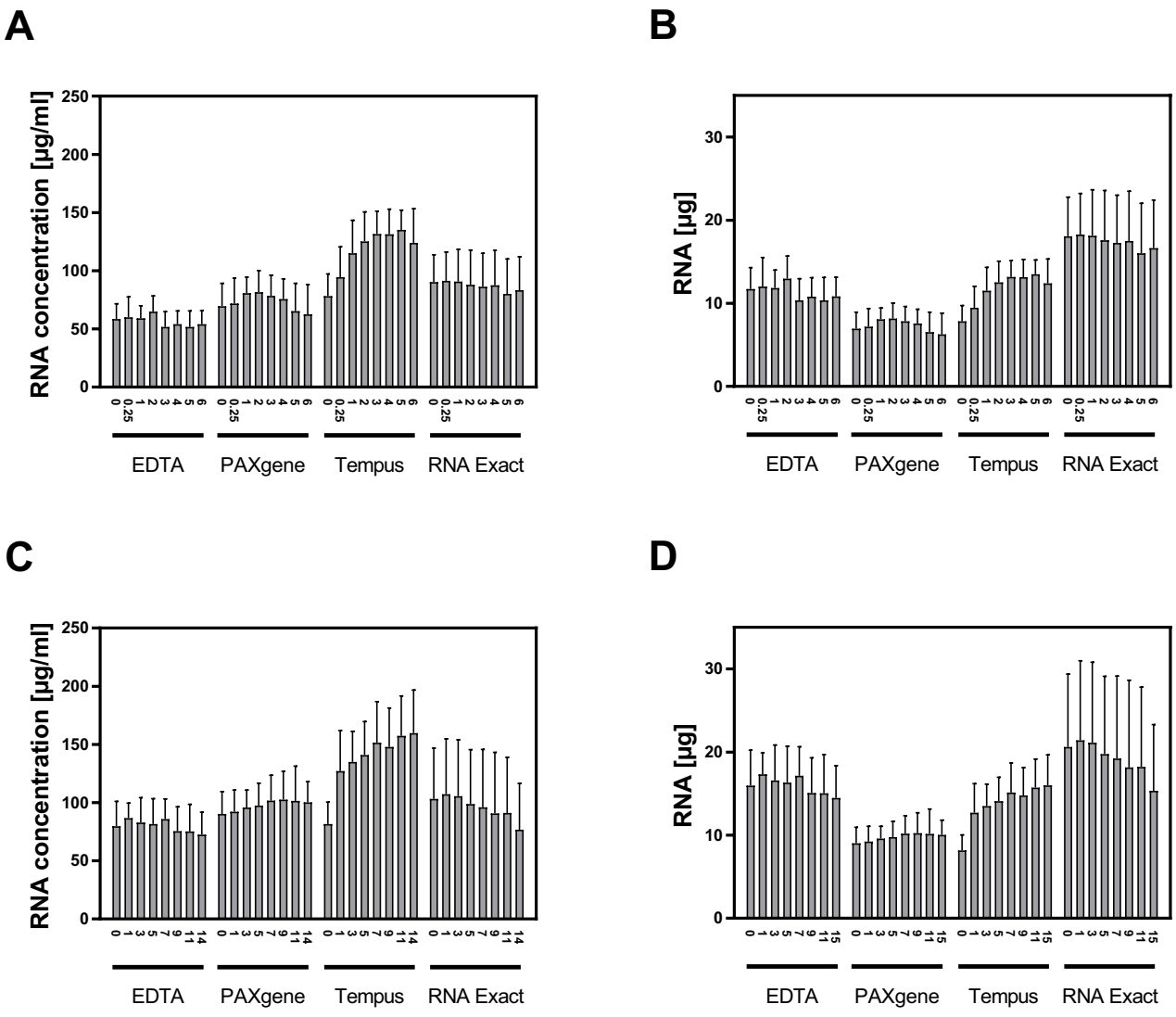

Figure 2: Stabilization yields different amounts of isolated RNA.
Blood was drawn into EDTA tubes, PAXgene™ tubes, Tempus™ tubes and the S-Monovette® RNA Exact and RNA was isolated after storing the samples at 22.5 °C (A and B) or at 8 °C (C and D)

Both PAXgene™ and Tempus™ offer their liquid biopsy products in combination with a proprietary RNA isolation system. For isolation, RNA is precipitated within the stabilized whole blood, spun down by high speed centrifugation, re-suspended and finally purified using silica matrix adsorption. These purification processes include several labor-intense steps, changing of vessels and heating of the samples (17 (Tempus™) and 21 "steps" (PAXgene™) respectively). In case of PAXgene™, a minimal incubation time of 2 h prior to RNA isolation is required, rendering this system the most time-intense (Figure 1). Furthermore, both systems require pellet formation. The need for high-speed centrifugation does not permit these systems to be integrated into automatized high-throughput routine diagnostics.

The S-Monovette® RNA Exact stabilizes RNA within whole blood and allows purification directly from the blood e.g. using common RNA isolation kits such as the Macherey-Nagel NucleoSpin® RNA Blood Midi Kit or the High Pure Viral Nucleic Acid Large Volume Kit (Roche Applied Science, Mannheim, Germany). RNA binds to the silica matrix directly from stabilized whole blood. Thus, there is no need for precipitation and centrifugation of RNA which allows this method to be integrated into automatized high-throughput systems (Figure 1).

RNA is stabilized and RNA integrity is maintained

After isolation of RNA from whole blood, we asked whether the different stabilization and purification methods result in similar quality of RNA.

Thus, we investigated the RIN (RNA-integrity). While RNA-integrity was equally high (RIN>8.8) in samples isolated at T0, RIN values decreased substantially over time in non-stabilized blood (EDTA) as well as in stabilized blood (PAXgene™ and S-Monovette® RNA Exact) when blood was stored at 22.5 °C. An even more prominent decrease of RNA-integrity was observed with the Tempus™ tubes (Figure 3A).

Upon storage of blood at 8 °C, RNA integrity of non-stabilized blood (EDTA) decreased; however to smaller extend than when stored at 22.5 °C (Figure 3B). Blood stabilized with the PAXgene™ tubes or the S-Monovette® RNA Exact yielded highly stable RNA throughout 14 days with RIN values >8. In contrast, RNA integrity within the Tempus™ tubes decreased substantially over time, yielding similar or even lower RIN values as non-stabilized blood (EDTA) at any time point (<7 at day 14).

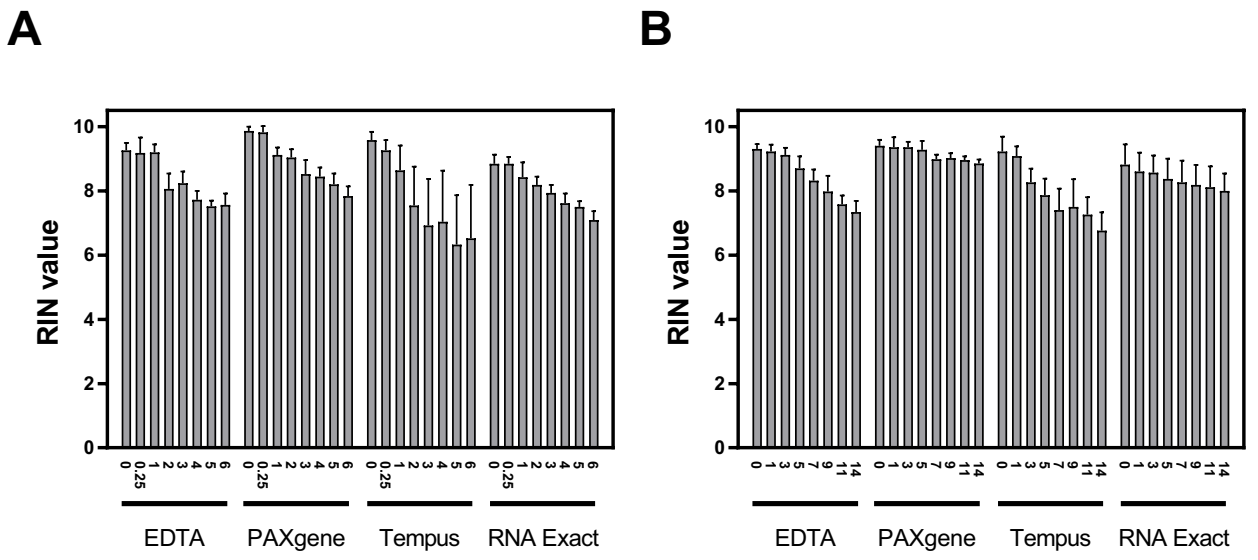

Figure 3: RNA integrity decreases in stored whole blood samples.
Blood was drawn into EDTA tubes, PAXgene™ tubes, Tempus™ tubes and the S-Monovette® RNA Exact. After storing whole blood at 22.5 °C (A) or 8 °C (B), RNA was isolated and RNA integrity was determined.

Stabilization of RNA conserves the expression levels of genes

RNA, and particularly mRNA, is rapidly synthesized and degraded. It is known that gene expression of e.g. IL-8 strongly increases within several hours after phlebotomy [1]. Thus, RNA stabilization has to act quickly in order to conserve the expression level of genes. Furthermore, RNA is degraded over time (see figure 3) and thus RNA-based diagnostics would be impaired. As a consequence, an RNA stabilizing agent has to act in a two-fold way: It has to stop cellular metabolism to avoid *de novo* synthesis of RNA after phlebotomy (1) and has to inhibit RNA degradation (2). We investigated whether RNA stabilization maintains RNA quality and quantity. To this end, we analyzed the expression levels of different genes (*FOS*, *FOXP3*, *IL1B*, *IL8* and *TNFRSF10C*) in samples from stabilized blood.

When blood samples were stored at 22.5 °C for six days, expression levels of IL8 were highly volatile in non-stabilized blood (EDTA). In addition, expression of genes such as *IL1B*, *FOS* and *TNFRSF10C* decreased over time, while expression of *FOXP3* was not markedly influenced in non-stabilized blood. The PAXgene™ system conserved gene expression levels of *FOXP3* and *TNFRSF10C* and – to some extent – expression levels of *IL1B* for six days. However, gene expression of *IL8* and *FOS* were not stabilized and showed a continuous decrease in their expression level.

The Tempus™ tubes and the S-Monovette® RNA Exact effectively stabilized gene expression levels of all five genes (*FOS*, *FOXP3*, *IL1B*, *IL8* and *TNFRSF10C*) for six days in blood samples stored at 22.5 °C. Surprisingly, with the Tempus™ system, it seems if there was an increase in gene expression of *IL1B*, *IL8* and – to a smaller extent – *TNFRSF10C* between day 0 and day 2, while no such effects were visible with the S-Monovette® RNA Exact (Figure 4). When blood samples were stored at 8 °C for 14 days, the resulting picture was similar to storage at 22.5 °C. In non-stabilized blood (EDTA), expression of *FOS*, *IL1B*, *IL8* and *TNFRSF10C* decreased over time, while expression of *FOXP3* was not influenced. The PAXgene™ system conserved gene expression levels of FOXP3, IL1B and TNFRSF10C for six days. However, gene expression of *IL8* and *FOS* were not stabilized and showed a continuous decrease in their expression level – the overall picture is very similar to the stabilization effect at 22.5 °C (Figure 5).

The Tempus™ tubes and the S-Monovette® RNA Exact effectively stabilized gene expression levels of all five genes (*FOS*, *FOXP3*, *IL1B*, *IL8* and *TNFRSF10C*) for 14 days in blood samples stored at 8 °C. However, with the Tempus™ tubes, there seems to be a sudden raise in gene expression for *FOS*, *IL1B*, *IL8* and *TNFRSF10C* after day 0 which stays at all further time points.

Discussion

RNA is degraded in EDTA-anticoagulated whole blood

After phlebotomy, there is a rapid decay of free RNA in EDTA-anticoagulated blood, representing a pronounced source of errors in RNA based diagnostics. Thus, there is a clear and present need for stabilization of RNA in whole blood, which is addressed with RNA stabilizing blood collection devices such as the PAXgene™ Blood RNA System. Previous studies have shown the PAXgene™ Blood RNA System to be an appropriate device for stabilizing RNA in whole blood and maintaining the amount of RNA in the blood sample for up to 5 days after phlebotomy [1, 28]. Here, we evaluated the amount of RNA that was isolated from whole blood stored in different blood collection systems.

From the S-Monovette® RNA Exact, the highest yield of RNA was obtained compared to non-stabilized blood (EDTA), PAXgene™ or Tempus™ blood collection tubes (Figure 2).

When blood is stabilized in the S-Monovette® RNA Exact at 22.5 °C for 6 days or at 8 °C for 14 days, the obtained RNA concentration and total RNA amount is higher than in non-stabilized blood (EDTA), PAXgene™ or Tempus™ tubes. The amount of RNA remains constant throughout the stabilization period in both the S-Monovette® RNA Exact and the PAXgene™ tubes at both 22.5 °C and 8 °C, indicating that there is no decay of RNA protection. In non-stabilized blood (EDTA) there is a clear, however not significant decrease of RNA amount over time. Surprisingly, the RNA yield from Tempus™ tubes is lowest at day 0 and increases until day 2 (22.5 °C) or day 7 (8 °C). This considerable increase in the RNA yield is, without doubt, not caused by *de novo* RNA synthesis within the blood collection tubes but is rather an effect of the stabilization principle of the Tempus™ preparation. The RNA isolation is dependent on precipitation with subsequent pellet formation which contains the nucleic acids. It seems that this precipitation is time-dependent and enhanced by lower temperatures. These findings indicate that isolation of the complete amount of RNA is not possible shortly after blood collection with Tempus™ tubes.

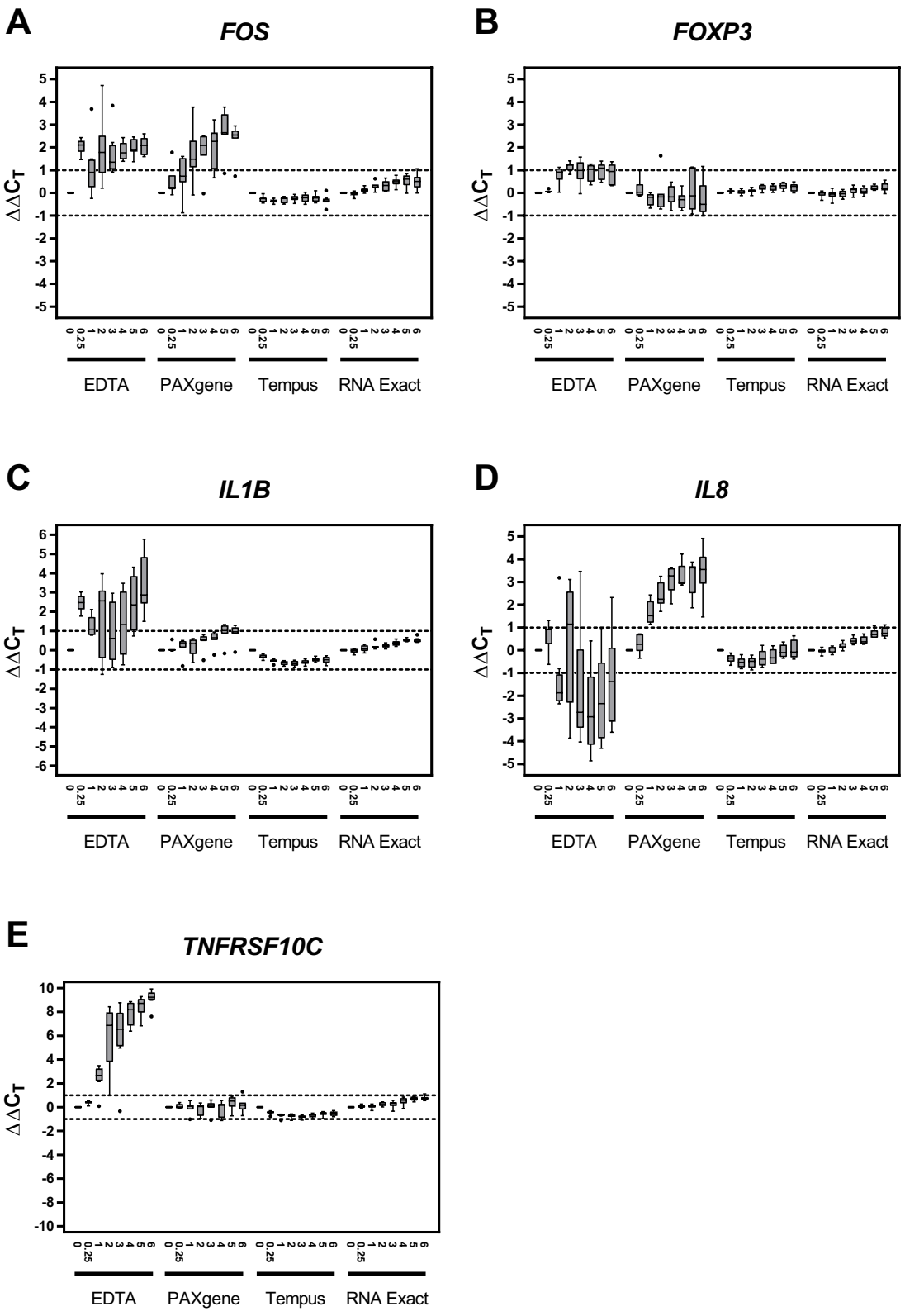

Figure 4: Stabilization of RNA conserves the expression level of genes (22.5 °C)
After isolation of RNA from stabilized whole blood stored for different time periods (days are indicated above the different blood collection tubes), cDNA was synthesized and expression levels of *FOS, FOXP3, IL1B, IL8* and *TNFRSF10C* were determined by qPCR.

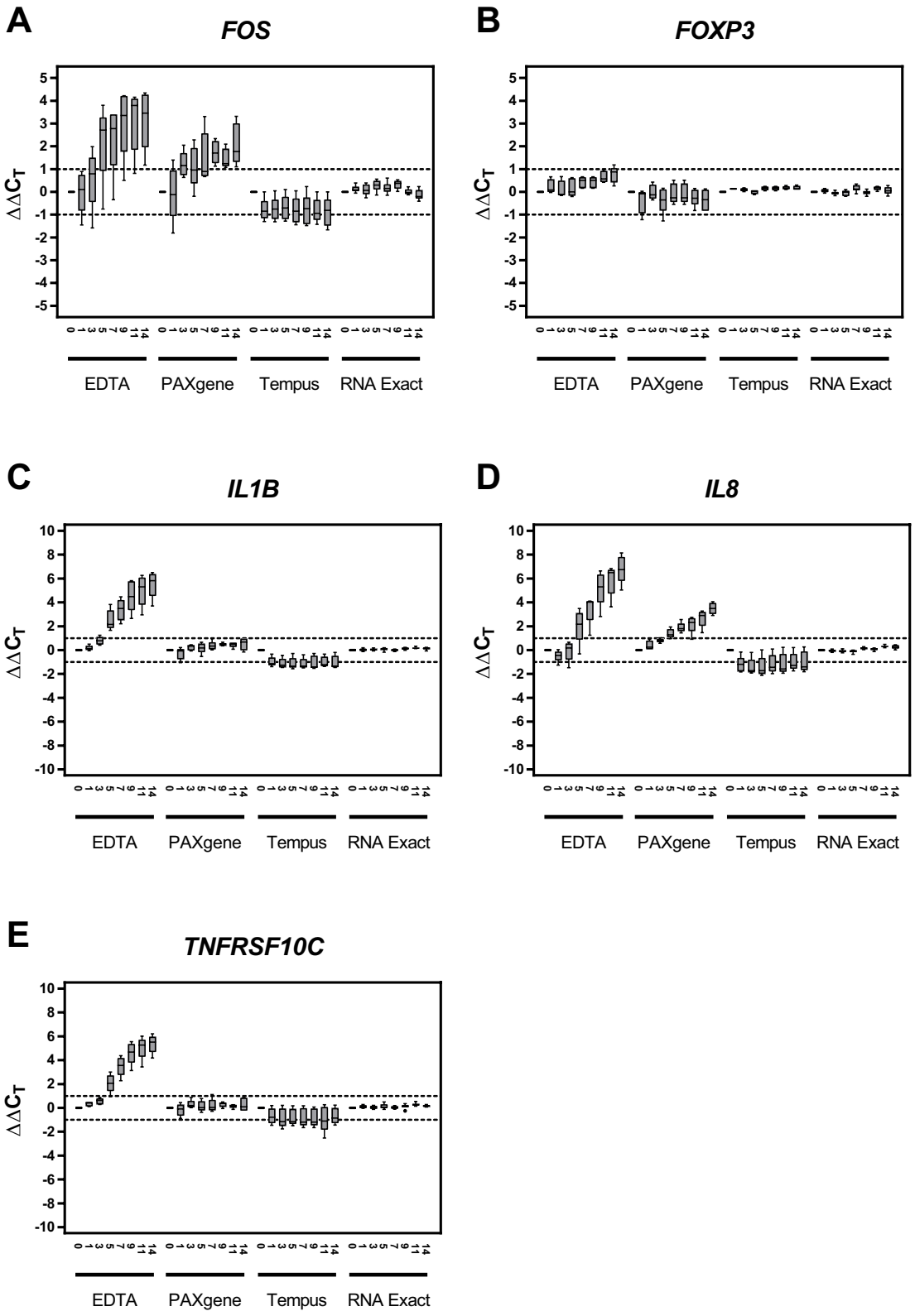

Figure 5: Stabilization of RNA conserves the expression level of genes (8 °C)
After isolation of RNA from stabilized whole blood stored for different time periods (days are indicated above the different blood collection tubes), cDNA was synthesized and expression levels of *FOS, FOXP3, IL1B, IL8* and *TNFRSF10C* were determined by qPCR.

The total amount of RNA is highest in the S-Monovette® RNA Exact followed in the Tempus™ system. However, the isolation efficiency is based on the combination of RNA stabilization and the subsequently applied isolation method. Considering that Tempus™ tubes contain 3 ml instead of up to 2.4 ml blood, the combination of RNA Exact and NucleoSpin RNA Blood Midi Kit yield the highest amount of RNA among all tested combinations. To this end, the direct isolation of RNA from the S-Monovette® RNA Exact is quicker and yields more DNA than the tested systems that comprise pellet formation and resuspension.

RNA is stabilized and RNA integrity is maintained

The assessment of the RNA integrity is based on a capillary electrophoresis with standardized consumables, an invariable protocol and an automatically applied algorithm calculating values between 0 and 10. The RIN value is regarded as a reliable and comparable size for evaluating the quality of RNA. Based on qRT-PCR analysis, RIN values higher than five have been described as good and values higher than eight as perfect total RNA quality [29]. The relevance of RIN in gene expression studies and a RIN cut-off of five was later ratified for several genes known to be induced or repressed by *ex vivo* blood handling [11]. However, RNA with RIN values lower than 5 should not generally be excluded from further analyses. The possible usage of RNA with lower RIN for distinct analyses has to be validated.

Within the S-Monovette® RNA Exact, RNA integrity is maintained to a similar extend when compared to PAXgene™ tubes and to a higher extend when compared to Tempus™ tubes.

The S-Monovette® RNA Exact does not show accelerated RNA decay (i.e. decreasing RIN values) throughout storage at 22.5 °C for 6 days or 14 days at 8 °C when compared to PAXgene™ tubes. Integrity of RNA isolated from blood stabilized in Tempus™ tubes is considerably lower, showing even lower RIN values than non-stabilized blood. However, the RIN values do not decrease below the cut-off of five in any sample.

Thus, with respect to RNA integrity, the S-Monovette® RNA Exact is comparable to the PAXgene™ device and yields higher RIN values than the Tempus™ blood collection tube.

Stabilization of RNA conserves the expression levels of genes

As stated above, previous studies have shown the PAXgene™ Blood RNA System to be an appropriate device for stabilizing RNA in whole blood. It was shown before that within the PAXgene™ device, the expression levels of several genes which are considered to be critical are stabilized for up to five days [1, 12]. Here, we evaluated the stabilization of gene expression in whole blood which was stored in different blood collection systems.

Gene expression is more efficiently stabilized by the S-Monovette® RNA Exact in comparison to PAXgene™ tubes and at least equally efficient to Tempus™ tubes. The S-Monovette® RNA Exact conserves the expression levels of all five genes investigated (FOS, FOXP3, IL1B, IL8 and TNFRSF10C) at both 22.5 °C and 8 °C. This is comparable to Tempus™ tubes. However, Tempus™ tubes show an increase in gene expression of IL1B, IL8 and TNFRSF10C between day 0 and day 2 ($\Delta\Delta CT<1$, see above), which is in accordance with increasing RNA levels in this period. Interestingly, this increase in expression is more prominent when samples were stored at 8 °C. As stated above, these findings may not reflect a failure of the stabilization performance but rather a kind of delay in the precipitation process which appears like altered gene expression upon comparing expression data from freshly isolated to stored samples. PAXgene™ tubes do not stabilize the expression levels of two out of five genes (FOS and IL8) at both 22.5 °C and 8 °C. The higher RIN we observed in RNA isolated with the PAXgene™ does no correspond to high performance in stabilizing mRNAs.

This means the chemical composition of the PAXgene™ tubes does not equally stabilize all mRNAs and that there is a kind of sequence dependency in stabilization. However, users will not be able to predict whether a gene of interest will be stabilized or not. An unequal stabilization of transcripts cannot be mathematically corrected via factoring of one or several housekeeping genes. Gene expression analyses and thus RNA based diagnostics will not yield reliable information under these conditions.

We can state that even under stabilizing conditions, half-life of mRNA species is not equal. For example, mRNA species like *IL-8*, an early marker for inflammatory processes, is highly susceptible to decomposition, even under stabilizing conditions, while mRNA of the transcription factor for regulatory T cells, *FOXP3*, is highly stable, even in non-stabilized blood.

Conclusion

Taken together, the S-Monovette® RNA Exact represents a novel and highly performant blood collection device for instant isolation and stabilization of RNA at various temperatures. Thus, whole blood for RNA analysis can be stored and transported within the S-Monovette® RNA Exact and RNA integrity and RNA concentrations are maintained. The stabilizing capacity of the S-Monovette® RNA Exact may even allow

critical transport conditions, e.g. uncooled transport by car, until RNA isolation and analysis by molecular diagnostic assays are performed. The stabilization performance of the S-Monovette® RNA Exact is more effective than that of widely used blood collection systems by PAXgene™ and Tempus™, which were tested in this study. Furthermore, highest amounts of RNA can be isolated from the S-Monovette® RNA Exact quickly and without time-intense centrifugation steps e.g. using the Macherey-Nagel NucleoSpin® RNA Blood Midi Kit. Thus, the S-Monovette® RNA Exact can be implemented into automated routine diagnostics. The S-Monovette® RNA Exact can be recommended for storage and transport of whole blood samples for subsequent RNA analysis for both clinically and scientific usage. With the development of the IVD certified S-Monovette® RNA Exact, SARSTEDT contributes to improvement of standardization in clinical diagnostics, biobanking and research.

Disclosures

The research is funded by SARSTEDT AG & Co. KG. JL,MK, DS, GE and RS are employees of SARSTEDT AG & Co. KG

References

1. Gunther, Kalle; Malentacchi, Francesca; Verderio, Paolo; Pizzamiglio, Sara; Ciniselli, Chiara Maura; Tichopad, Ales et al. (2012): Implementation of a proficiency testing for the assessment of the preanalytical phase of blood samples used for RNA based analysis. In: Clinica chimica acta; international journal of clinical chemistry 413 (7-8), S. 779–786.
2. Morton, Charles Oliver; Luca, Antonella de; Romani, Luigina; Rogers, Thomas Richard (2012): RT-qPCR detection of Aspergillus fumigatus RNA in vitro and in a murine model of invasive aspergillosis utilizing the PAXgene(R) and Tempus RNA stabilization systems. In: Medical mycology 50 (6), S. 661–666. DOI: 10.3109/13693786.2011.652200.
3. Whitney, Adeline R.; Diehn, Maximilian; Popper, Stephen J.; Alizadeh, Ash A.; Boldrick, Jennifer C.; Relman, David A.; Brown, Patrick O. (2003): Individuality and variation in gene expression patterns in human blood. In: Proceedings of the National Academy of Sciences 100 (4), S. 1896–1901. DOI: 10.1073/pnas.252784499.
4. Miller, Todd E.; You, Lijing; Myerburg, Robert J.; Benke, Paul J.; Bishopric, Nanette H. (2007): Whole blood RNA offers a rapid, comprehensive approach to genetic diagnosis of cardiovascular diseases. In: Genetics in medicine : official journal of the American College of Medical Genetics 9 (1), S. 23–33.
5. Wang, S.-W.; Pawlowski, J.; Wathen, S. T.; Kinney, S. D.; Lichenstein, H. S.; Manthey, C. L. (1999): Cytokine mRNA decay is accelerated by an inhibitor of p38-mitogen-activated protein kinase. In: Inflammation Research 48 (10), S. 533–538. DOI: 10.1007/s000110050499.
6. Das, Kausik; Norton, Sheila E.; Alt, Jodi R.; Krzyzanowski, Gary D.; Williams, Thomas L.; Fernando, M. Rohan (2014): Stabilization of cellular RNA in blood during storage at room temperature: a comparison of cell-free RNA BCT((R)) with K3EDTA tubes. In: Molecular diagnosis & therapy 18 (6), S. 647–653. DOI: 10.1007/s40291-014-0118-z.
7. Fraser Callum G. (2005): Inherent biological variation and reference values (Clinical Chemistry and Laboratory Medicine (CCLM), 42), 2005.
8. Opitz, Lennart; Salinas-Riester, Gabriela; Grade, Marian; Jung, Klaus; Jo, Peter; Emons, Georg et al. (2010): Impact of RNA degradation on gene expression profiling. In: BMC medical genomics 3, S. 36. DOI: 10.1186/1755-8794-3-36.
9. Benoy Ina H.; Elst Hilde; Van Dam Peter; Scharpé Simon; Van Marck Eric; Vermeulen Peter B.; Dirix Luc Y. (2011): Detection of circulating tumour cells in blood by quantitative real-time RT-PCR: effect of pre-analytical time (Clinical Chemistry and Laboratory Medicine (CCLM), 44), 2011.
10. Tanner, M. A.; Berk, L. S.; Felten, D. L.; Blidy, A. D.; Bit, S. L.; Ruff, D. W. (2002): Substantial changes in gene expression level due to the storage temperature and storage duration of human whole blood. In: Clinical and laboratory haematology 24 (6), S. 337–341.
11. Pazzagli, M.; Malentacchi, F.; Simi, L.; Orlando, C.; Wyrich, R.; Gunther, K. et al. (2013): SPIDIA-RNA: first external quality assessment for the pre-analytical phase of blood samples used for RNA based analyses. In: Methods (San Diego, Calif.) 59 (1), S. 20–31. DOI: 10.1016/j.ymeth.2012.10.007.

12. Malentacchi, Francesca; Pazzagli, Mario; Simi, Lisa; Orlando, Claudio; Wyrich, Ralf; Gunther, Kalle et al. (2014): SPIDIA-RNA: second external quality assessment for the pre-analytical phase of blood samples used for RNA based analyses. In: PloS one 9 (11), S. e112293. DOI: 10.1371/journal.pone.0112293.
13. Matheson, Loren A.; Duong, Trang T.; Rosenberg, Alan M.; Yeung, Rae S.M. (2008): Assessment of sample collection and storage methods for multicenter immunologic research in children. In: Journal of immunological methods 339 (1), S. 82–89. DOI: 10.1016/j.jim.2008.08.003.
14. Rainen, Lynne; Oelmueller, Uwe; Jurgensen, Stewart; Wyrich, Ralf; Ballas, Cynthia; Schram, Jim et al. (2002): Stabilization of mRNA expression in whole blood samples. In: Clinical chemistry 48 (11), S. 1883–1890.
15. Weber, Daniel Gilbert; Casjens, Swaantje; Rozynek, Peter; Lehnert, Martin; Zilch-Schoneweis, Sandra; Bryk, Oleksandr et al. (2010): Assessment of mRNA and microRNA Stabilization in Peripheral Human Blood for Multicenter Studies and Biobanks. In: Biomarker insights 5, S. 95–102.
16. Thach, Dzung C.; Lin, Baochuan; Walter, Elizabeth; Kruzelock, Rusty; Rowley, Robb K.; Tibbetts, Clark; Stenger, David A. (2003): Assessment of two methods for handling blood in collection tubes with RNA stabilizing agent for surveillance of gene expression profiles with high density microarrays. In: Journal of immunological methods 283 (1), S. 269–279. DOI: 10.1016/j.jim.2003.10.004.
17. Aarem, Jeanette; Brunborg, Gunnar; Aas, Kaja K.; Harbak, Kari; Taipale, Miia M.; Magnus, Per et al. (2016): Comparison of blood RNA isolation methods from samples stabilized in Tempus tubes and stored at a large human biobank. In: BMC research notes 9 (1), S. 430. DOI: 10.1186/s13104-016-2224-y.
18. Feddersen, Søren; Bastholt, Lars; Pedersen, Susanne M. (2017): Stabilization of circulating thyroglobulin mRNA transcripts in patients treated for differentiated thyroid carcinoma. In: Annals of Clinical Biochemistry 54 (5), S. 558–566. DOI: 10.1177/0004563216671538.
19. Kim, Jin-Hee; Jin, Hyeon-Ok; Park, Jin-Ah; Chang, Yoon Hwan; Hong, Young Jun; Lee, Jin Kyung (2014): Comparison of three different kits for extraction of high-quality RNA from frozen blood. In: SpringerPlus 3, S. 76. DOI: 10.1186/2193-1801-3-76.
20. Øvstebø, Reidun; Lande, Knut; Kierulf, Peter; Haug, Kari Bente Foss (2007): Quantification of relative changes in specific mRNAs from frozen whole blood - methodological considerations and clinical implications. In: Clinical chemistry and laboratory medicine 45 (2), S. 171–176.
21. Nikula, Tuomas; Mykkänen, Juha; Simell, Olli; Lahesmaa, Riitta (2013): Genome-wide comparison of two RNA-stabilizing reagents for transcriptional profiling of peripheral blood. In: Translational research : the journal of laboratory and clinical medicine 161 (3), S. 181–188.
22. Kågedal, Bertil; Lindqvist, Malin; Farnebäck, Malin; Lenner, Liselotte; Peterson, Curt (2005): Failure of the PAXgene Blood RNA System to maintain mRNA stability in whole blood. In: Clinical chemistry and laboratory medicine 43 (11), S. 1190–1192. DOI: 10.1515/CCLM.2005.206.
23. Tsui, Nancy B.Y.; Ng, Enders K.O.; Lo, Y. DennisM. (2002): Stability of Endogenous and Added RNA in Blood Specimens, Serum, and Plasma. In: Clinical chemistry 48 (10), S. 1647–1653.
24. Bowen, Raffick A.R.; Adcock, Dorothy M. (2016): Blood collection tubes as medical devices: The potential to affect assays and proposed verification and validation processes for the clinical laboratory. In: Preanalytical aspects of laboratory testing 49 (18), S. 1321–1330. DOI: 10.1016/j.clinbiochem.2016.10.004.
25. Zhang, X.; Ding, L.; Sandford, A. J. (2005): Selection of reference genes for gene expression studies in human neutrophils by real-time PCR. In: BMC molecular biology 6, S. 4. DOI: 10.1186/1471-2199-6-4.
26. Sun, Yuan; Li, Yan; Luo, Dianzhong; Liao, D. Joshua (2012): Pseudogenes as weaknesses of ACTB (Actb) and GAPDH (Gapdh) used as reference genes in reverse transcription and polymerase chain reactions. In: PloS one 7 (8), S. e41659. DOI: 10.1371/journal.pone.0041659.
27. Vandesompele, Jo; Preter, Katleen de; Pattyn, Filip; Poppe, Bruce; van Roy, Nadine; Paepe, Anne de; Speleman, Frank (2002): Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. In: Genome biology 3 (7), S. RESEARCH0034.
28. Langelaan, M. L. P.; Bock, Dylus E.; Jongen, B.; Mertens, A. A. M.; Raijmakers, M. T. M. (2014): Improved pre-analytical process for RNA isolation from whole blood samples. In: Ned Tijdschr Klin Chem Labgeneesk (39), S. 164–165.
29. Fleige, Simone; Pfaffl, Michael W. (2006): RNA integrity and the effect on the real-time qRT-PCR performance. In: Molecular aspects of medicine 27 (2-3), S. 126–139. DOI: 10.1016/j.mam.2005.12.003.

For additional product or technical information, please e-mail us at marketing@sarstedt.com or visit www.sarstedt.com.

SARSTEDT AG & Co. KG
Sarstedtstraße 1
D-51588 Nümbrecht
Germany

www.sarstedt.com
info@sarstedt.com

AN_007_0123