Simultaneous extraction of mRNA and microRNA with the MagMAX™ mirVana™ Total RNA Isolation Kit from S-Monovette® RNA Exact

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INTRODUCTION

S-Monovette® RNA Exact is a collection tube that is used to preserve RNA molecules in biological samples for diagnostic or research purposes. RNA, or ribonucleic acid, is a molecule that plays a crucial role in gene expression and regulation, and is often used as a biomarker for various diseases and physiological conditions.
During collection and transportation of biological samples in anticoagulated for example EDTA containing blood tubes, gene expression levels cannot be conserved (Das et al. 2014; Fraser Callum G. 2005). The RNA can be easily degraded *ex vivo* by various factors such as nucleases and heat (Opitz et al. 2010; Benoy Ina H. et al. 2011). Gene expression is heavily dysregulated because of the phlebotomy itself and by causes after phlebotomy, like sample storage in anticoagulating agents or hemolysis (Tanner et al. 2002), or simply surface contact of the lymphocytes to the tube walls.
This compromises the accuracy of analysis and can lead to false-negative results in diagnostic tests. In need for quality standards and improvement of pre-analytic procedures the SPIDIA project (Standardization and improvement of generic pre-analytical tools and procedures for *in vitro* diagnostics), highlighted 2014 the importance of stabilization of RNA right after phlebotomy for reliable gene expression analysis (Malentacchi et al. 2014).
RNA stabilizing blood collection tubes are designed to prevent RNA degradation by providing a stable environment for all RNA molecules to be preserved until they can be analyzed in the laboratory (Matheson et al. 2008). The widely used PAXgene® Blood RNA System comprises one of the described RNA stabilizing tubes. The system consists of a blood collection tube (PAXgene® Blood RNA Tube) and different RNA isolation kits, including a kit for total RNA > 18 nucleotides (PAXgene® Blood miRNA Kit). Therefore we use this as a comparison system to our S-Monovette® RNA Exact with the kit described.
Overall, the use of S-Monovette® RNA Exact is very beneficial in diagnostics, as it ensures the integrity of RNA samples and helps to warrant the accuracy of diagnostic tests. Directly after phlebotomy the sample is immediately lysed after contact with the preparation solution. In addition, the S-Monovette® RNA Exact contains a proprietary formulation which directly adjusts the binding conditions for many silica-membrane or magnetic bead based RNA extraction systems. This leads to time saving isolation protocols and minimizes hands on time. RNA can be isolated from the S-Monovette® RNA Exact with various different manual and automated isolation kits (see also instructions for use).
Here we describe the isolation with the MagMAX™ mirVana™ Total RNA Isolation Kit (REF A27828) using the KingFisher™ Flex Magnetic Particle Processor 96 DW.

Materials and Methods

Blood sample collection and storage:

Blood from 11 healthy donors was drawn into S-Monovette® RNA Exact blood collection tubes (REF 01.2048.001, Sarstedt AG & Co., Nümbrecht, Germany) and blood from 9 healthy donors was drawn into PAXgene® Blood RNA Tubes (PreAnalytiX GmbH, Hombrechtikon, Switzerland).
PAXgene® Blood RNA Tube were stored upright at room temperature (18 °C – 25 °C) for a minimum of 2 hours before transferring to a freezer (-80 °C) at day 0 or stored for 3 days at 25 °C (D3) and then transferred to the freezer. S-Monovette® RNA Exact samples were frozen at -80 °C within 30 minutes after blood collection for D0 samples or after storage of the blood collection tube for 3 days at 25 °C (D3) until isolation.

RNA isolation:

RNA was isolated from the complete PAXgene® Blood RNA Tube according to manufacturer's instructions at different time points (D0 and D3). From S-Monovette® RNA Exact 600 µl lysed Blood at different time points (D0 and D3) was used for isolation with the MagMAX™mirVana™ Total RNA Isolation Kit on the Kingfisher™ Flex 96-well format. Isolation was performed according to an adapted MagMAX™ mirVana ™ Total RNA Isolation Kit manual, taking into account that the RNA stabilizer of the S-Monovette® RNA Exact already adjusts lysis/binding conditions as well as the input dependent scaling of the volumes.
Detailed protocols and scripts for RNA isolation from S-Monovette® RNA-Exact are provided later in this application note. The maximum isolation volume with the 96 KF Deep-well magnetic head is around 900 µl of lysed RNA Exact sample. Further upscaling is possible with positioning more than one well per sample or using the KF 24 Deep-well magnetic head.

RNA analysis:
RNA integrity for the S-Monovette® RNA Exact and PAXgene® samples was measured with a Bioanalyzer 2100 (Agilent, Waldbronn, Germany) using RNA Nano 6000 Chips (Agilent REF 5067-1511).

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 rewriting of fragmented mRNA lacking a poly(A) tail.

To investigate RNA preservation over time, quantitative real-time PCRs of six genes were conducted, including two housekeeping genes. Because traces of resting DNA always can remain in the sample, we designed primers located directly on exon/intron boundaries or primers spanning exon/intron boundaries to prevent signals derived from remaining DNA. Templates have been located near the 5´end of the mRNA and the reverse transcription is always run with polyT-primers to ensure to transcribe only full-length mRNA molecules. All primers were synthesized by Biospring GmbH (Frankfurt, Germany).

Table 1: Primers used for the analysis of gene expression after RNA isolation with MagMAX™ mirVana™ Total RNA Isolation Kit from S-Monovette® RNA Exact.

gene	Accession No.	primer	Amplicon length
hydroxymethylbilane synthase (PBGD)	NM_000190.3	PBGD_fw CTGGTAACGGCAATGCGGC PBGD_377_rv TCTCTCCAATCTTAGAGAGTGCAG	216 bp
guanine nucleotide binding protein, beta polypeptide 2-like 1 (GNB2L1)	NM_006098.4	GNB2L1_43 GAGTGTGGCCTTCTCCTCTG GNB2L1_656rv GCTTGCAGTTAGCCAGGTTC	224 bp
chemokine ligand 8 (IL-8)	NM_000584.3	IL-8_126fw GGAAGGAACCATCTCACTGTG IL-8_276rv GGAGTATGTCTTTATGCACTGAC	151 bp
Interleukin 1, beta (IL-1β)	NM_000576.2	IL-1B_4fw AAACCTCTTCGAGGCACAAGG IL-1B_201rv GTCCTGGAAGGAGCACTTCATC	198 bp
FBJ murine osteosarcoma viral oncogene homolog (FOS)	NM_005252.3	FOS_336fw TCAACGCGCAGGACTTCTGC FOS_688rv TCTCCGCTTGGAGTGTATCAGTC	375 bp
Tumor necrosis factor receptor superfamily member 10c (TNFRSF10C)	NM_003841.3	TNFRSF10C_318fw ATCCCCAAGACCCTAAAGTTCG TNFRSF10C_480rv GAGATCCTGCTGGACACTCCTC	163 bp

For miRNA analysis the RNA was reversely transcribed into cDNA using the TaqMan™ MicroRNA Kit Reverse Transcription Kit (REF4366596, Applied Biosystems™) according to manufacturer's instructions including the primers for the desired TaqMan® MicroRNA Assay. PCR amplification was performed for three different TaqMan® MicroRNA Assays (hsa-let-7g-5p, hsa-miR-16-5p, hsa-miR-21-5p) according to the manufacturer's instructions.

Results & Discussion

Capillary electrophoresis was performed to investigate the RNA integrity. The electropherograms show two defined peaks of ribosomal RNA in all samples. The S-Monovette® RNA Exact Day 0 samples show RIN values of 9,6 and 9,4. After 3 days the RIN value decreases slightly to 7,6 and 7,7. For the PAXgene® samples the electropherograms show the same two peaks of ribosomal RNA in all samples. The RIN values are 9,8 and 9,5 for Day 0. After 3 days of storage the RIN values decrease slightly to 8,3 and 7,6. Measured by the RIN value, which mainly evaluates the structural RNA's, both isolation systems result in intact samples (Figure 1).

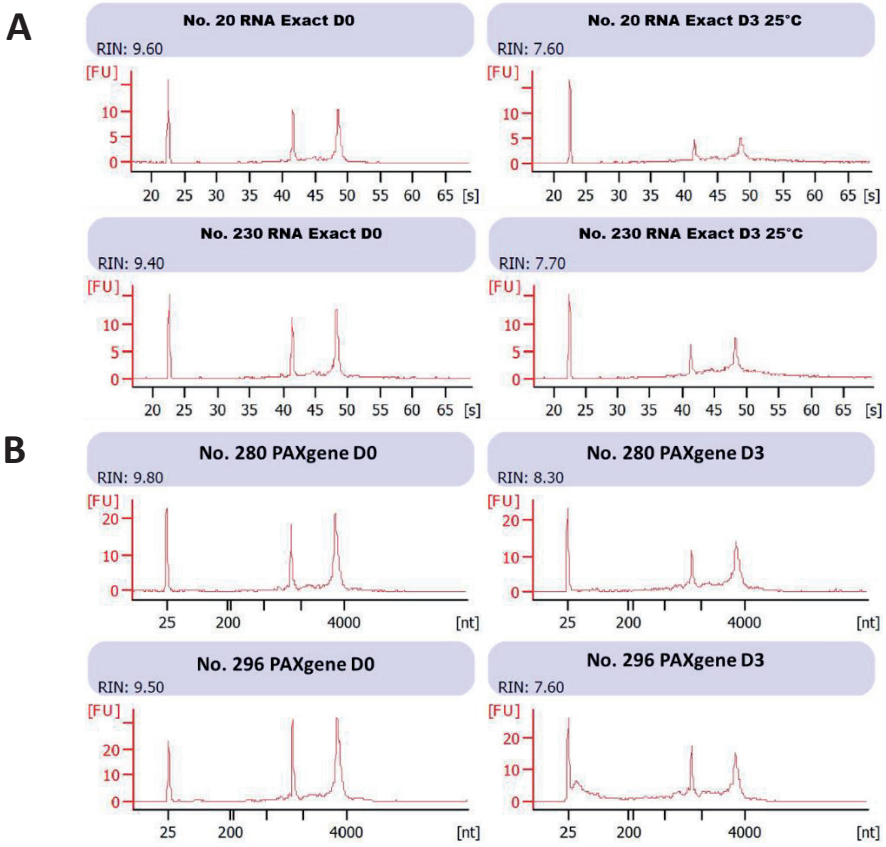

Figure 1: RNA integrity was measured with a Bioanalyzer 2100 (Agilent, Waldbronn, Germany) using RNA Nano 6000 Chips (Agilent REF 5067-1511). Data is shown exemplary for two donors each. (A) Isolation from the S-Monovette® RNA Exact were performed according to the protocol appended and show RIN values of 9,6 and 9,4 for day 0 or 7,6 and 7,7 for day 3 (B). The PAXgene® samples show RIN values of 9,8 and 9,5 for day 0 or 8,3 and 7,6 for day 3.

mRNA Analysis

Reverse transcription and real-time PCRs of the isolated samples with four different genes and two housekeeping genes were performed. Compared to the PAXgene® system the results from the S-Monovette® RNA Exact show comparable C_T values, although the processed sample quantity was only one tenth (arithmetically around 3 C_T values difference). Moreover, the results after three days show significantly better stabilization compared to PAXgene® system. The C_T values of the different time points and systems are shown in Figure 2.

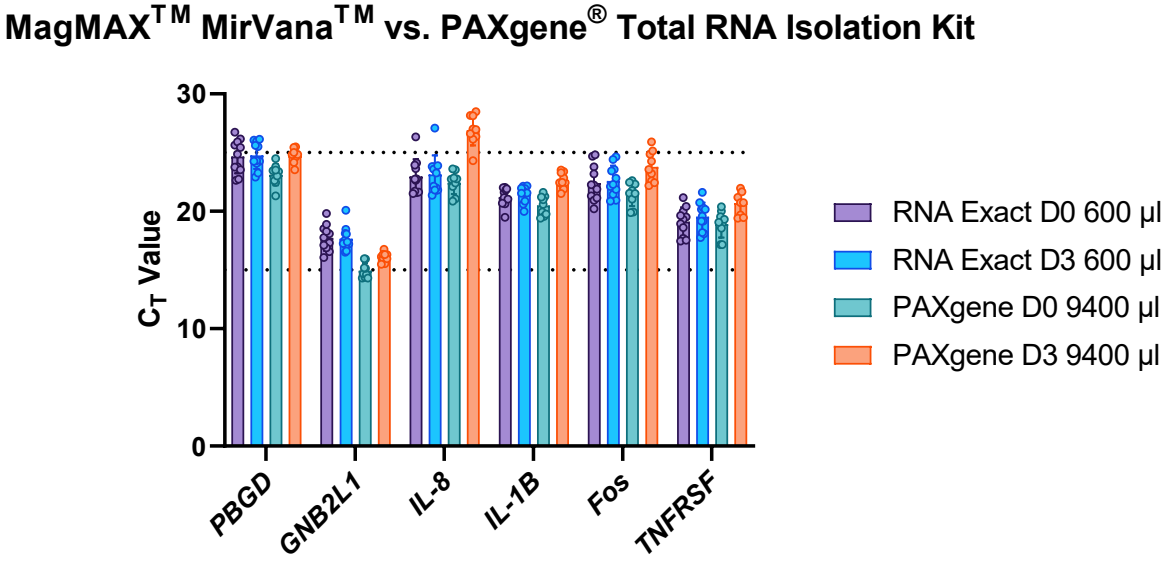

Figure 2: PCR Results after isolation with MagMAX™ mirVana™ Total RNA Isolation Kit. The C_T values of 9 or 11 independent donors were plotted here. The mean values and standard deviations are shown. Two housekeeping genes and four other genes were evaluated and the results were analyzed after RNA isolation from PAXgene® Blood tubes or from the S-Monovette® RNA Exact, either directly at day 0 or after 3 days of storage at room temperature. The dotted lines mark C_T values 15 and 25 for orientation purposes.

Figure 3 shows the $\Delta\Delta C_T$ values calculated to the C_T value from the day 0 samples of both systems after correction of the isolation efficiency via the C_T-values of the housekeeping genes. With the S-Monovette® RNA Exact the transcript level of the four selected genes keep stable over the desired three days at 25 °C, while in contrast the transcript levels of three out of the four genes change drastically in the PAXgene® system at day 3 compared to day 0.
The significantly enhanced stabilization performance of the S-Monovette® RNA Exact becomes evident in this context.

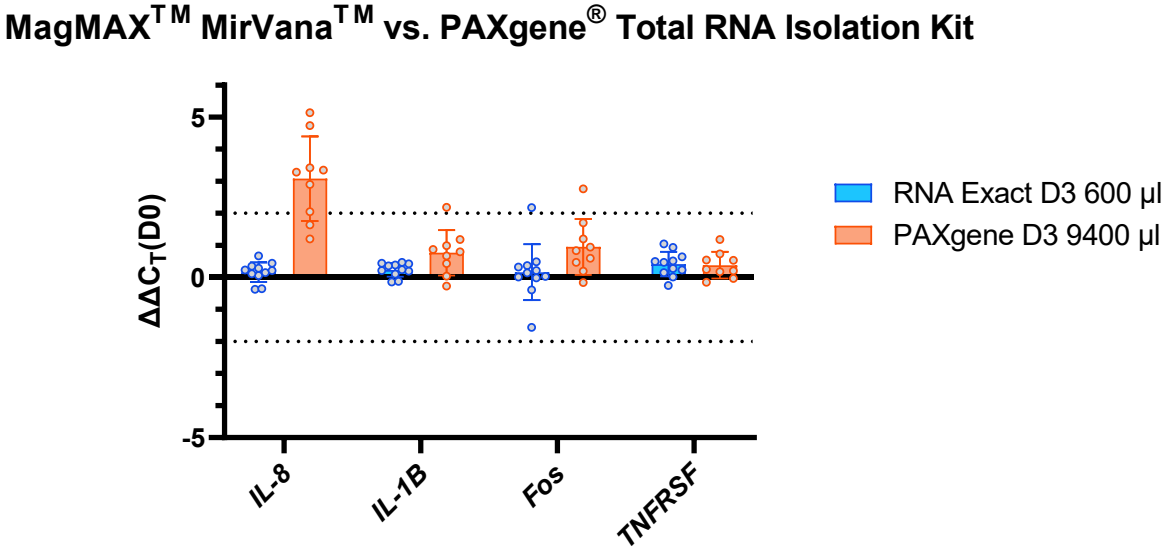

Figure 3: Calculation of the $\Delta\Delta C_T$ values for 9 or 11 different donors compared to the Day 0 samples. Depicted are the single values and the mean values with the standard deviation for 4 genes. The dotted lines show $\Delta\Delta C_T$ values -2 and 2 for orientation.

miRNA Analysis

Additional to mRNA analysis we also tested for microRNA isolation by analyzing three different micro RNAs (miRNAs) in the S-Monovette® RNA Exact samples compared to the PAXgene® system. The C_T-values of the selected miRNAs obtained with the MirVana/RNA Exact system on fresh samples (D0) are higher compared to the corresponding PAXgene® samples but in contrast to the PAXgene® samples the C_T values keep stable after storing the blood collection tubes for three days (Figure 4).

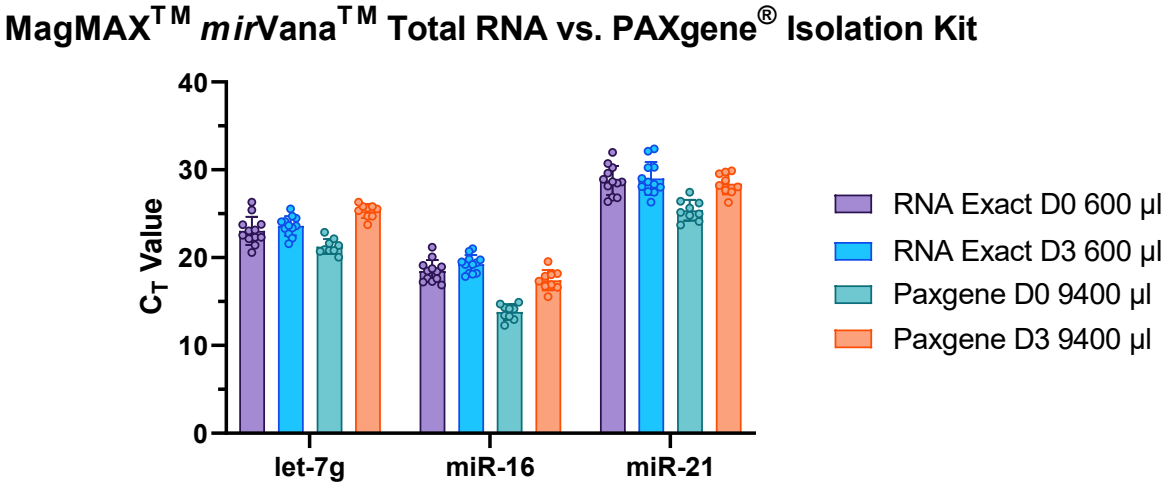

Figure 4: PCR Results for three miRNAs after isolation with MagMAX™ mirVana™ Total RNA Isolation Kit or the PAXgene® Blood miRNA Kit. The C_T values of 11 or 9 independent donors are plotted here. The mean values and standard deviations are shown. Three different micro RNAs were evaluated with the PAXgene® Blood miRNA Kit or from the S-Monovette® RNA Exact with the MagMAX™ Total RNA Isolation Kit either directly or after 3 days of storage at 25 °C.

After calculating the Δ of the C_T-values D3/D0 the better stabilization performance of the S-Monovette® RNA Exact becomes even more obvious. Without the correction by housekeeping genes as done for the mRNA analysis the ΔC_T is much more prominent. The miRNA content of the PAXgene® tubes is highly reduced after storage of three days for all three tested miRNAs (Figure 5).

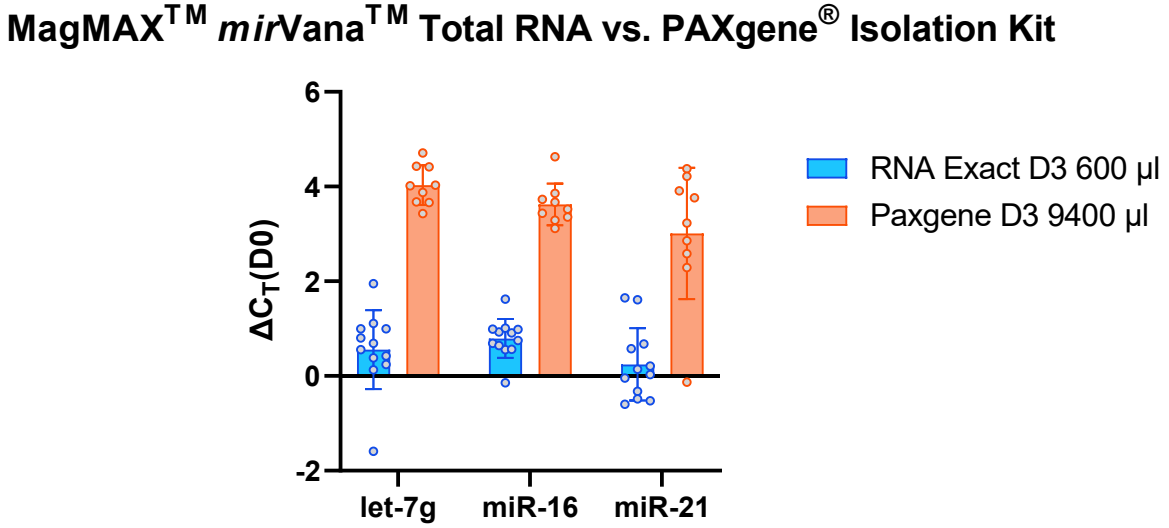

Figure 5: Calculation of the delta C_T values compared to Day 0 from the S-Monovette® RNA Exact or the PAXgene® blood Tube. Either 11 or 9 independent donors are plotted. Depicted are the single values and the mean values with the standard deviation for 3 miRNAs.

Summary

In this application note we show the compatibility of S-Monovette® RNA Exact stabilized samples with MagMAX™ mirvana™ Total RNA Isolation Kit (REF A27828) from Applied Biosystems™. With the RNA Isolation Kit, originally developed for serum/plasma, whole blood, tissue, cell culture or urine, high quality mRNA and miRNA could be isolated using lysed blood generated from S-Monovette® RNA Exact. Essential protocol simplifications are possible due to the already performed lysis and stabilization in the sample tubes.

Using the S-Monovette® RNA Exact, the RNA is not only stabilized for up to 3 days at room temperature, but also the isolation is much faster, because all pre-incubation steps can be outsourced to the KingFisher Flex™ device. The lysis carried out in the S-Monovette® allows the sample to be transferred directly into the deep well Plate. This also significantly simplifies the standard protocol of the MagMAX™ mirvana™ Total RNA Isolation Kit when blood is processed (Figure 6).

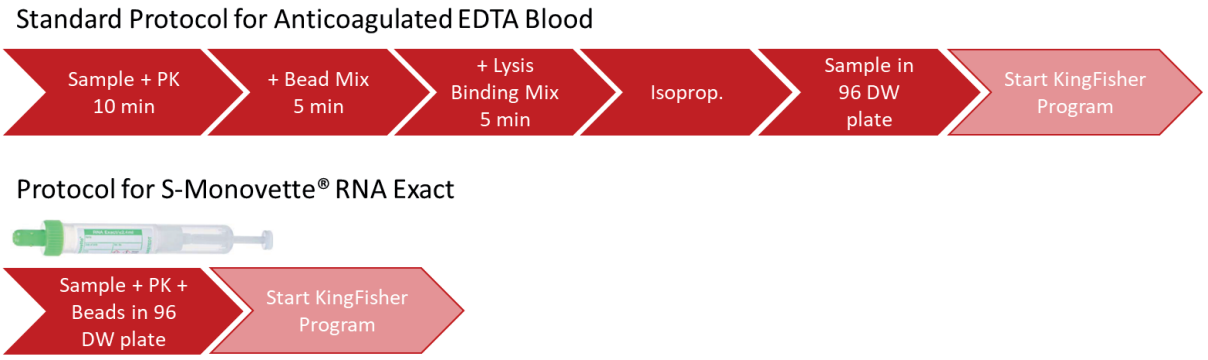

Figure 6: The figure shows the time saved when using the S-Monovette® RNA Exact instead of the standard sample input like anticoagulated EDTA blood with the King Fisher™ Flex.

The isolation efficiency of the combination of MagMAX™ MirVana™ RNA Kit with the S-Monovette® RNA Exact is highly superior to the established PAXgene® RNA stabilization and isolation system (manual protocol). Comparable C_T-values were obtained even with the use of the volume limited 96 deep well format.

Dependent on the individual needs the user has the possibility for upscaling up to 900 µl or by using replicates or the 24 well format, additionally there is enough to freeze a reserve sample.

DETAILED ISOLATION PROTOCOL

1. Prepare RNA Binding Beads and TURBO DNase™ Solution

Component	Volume per sample/well
MagMAX™ TURBO DNase™ Buffer	96 µl
TURBO DNase™	4 µl

Component	Volume per sample/well
RNA Binding Beads	30 µl
Lysis/Binding Enhancer	

2. Prepare Procession plates described in the following table

Table 2 Processing plates

Plate ID	Plate type	Reagent	Volume per well
Wash 1	96 DW plate	Wash 1	450 µl
Wash 2		Wash 2	450 µl
DNase Plate		TURBO DNase Mix	100 µl
Wash 3		Wash 2	450 µl
Wash 4		Wash 2	450 µl
Elution Plate		Elution buffer	75 µl
Tip		Place a 96 tip comb (Sarstedt, REF 82.1972.800) for deep-well magnets in a 96 DW plate (Sarstedt, REF 82.1972)	

3. Prepare Sample Plate (e.g. 96 DW Plate Sarstedt REF 82.1972)

- Submit **600 µl lysed blood** to each well
- Add **15 µl Proteinase K**
- Add **60 µl Binding Beads Mix**

4. Wash, rebind, and elute the RNA

Ensure that the instrument is set up for processing with the deep well magnetic head and select the program on the instrument.

A27828_FLEX_Biofluids_600µl_RNA Exact on KingFisher™ Flex Magnetic Particle Processor

Start the run and load the prepared processing plates in their positions when prompted by the instrument (see Table 2).

Load the sample plate when prompted by the instrument (30 – 35 minutes after the initial start):

a. Remove the DNase Plate from the instrument.
b. Add 100 µl of Rebinding Buffer and 200 µl of isopropanol to each sample well.

Add Rebinding Buffer and isopropanol immediately after the prompt, to prevent excessive drying of any beads that are still captured on the Tip Comb.

IMPORTANT! Do not pre-mix the Rebinding Buffer and isopropanol. Add them separately to the samples.

c. Load the DNase plate back onto the instrument, and press Start.

At the end of the run (approximately 60 minutes after the initial start), remove the plate from the instrument and seal immediately with a new adhesive film (e.g. SARSTEDT PCR Foil REF 95.1994).
(Optional) Eluates can be transferred to a storage plate or micro screw cap tubes after collection.

IMPORTANT! Do not allow the purified samples to sit uncovered at room temperature for more than 10 minutes, to prevent evaporation and contamination.

The purified samples are ready for immediate use. Alternatively, store them: On ice for up to 8 hours or at -80 °C for long-term storage.

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