Simultaneous extraction of mRNA and microRNA with the Maxwell® CSC RNA Blood 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 Maxwell® CSC RNA Blood Kit (REF AS1410) using the Maxwell® CSC Instrument (AS6000).

Materials and Methods

Blood sample collection and storage:

Blood from 10 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 1000 µl lysed Blood at different time points (D0 and D3) was used for isolation with the Maxwell® CSC RNA Blood Kit (REF AS1410) using the Maxwell® CSC Instrument (AS6000). Isolation was performed according to an adapted Maxwell® CSC RNA Blood Kit (REF AS1410) manual, taking into account that the RNA stabilizer of the S-Monovette® RNA Exact already adjusts Lysis/Binding conditions.
Detailed protocols and scripts for RNA isolation from S-Monovette® RNA-Exact are provided later in this application note.

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 Maxwell® CSC RNA Blood Kit (REF AS1410) 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 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 measured samples. The S-Monovette® RNA Exact Day 0 samples show RIN values of and 9,2 and 9,4. After 3 days the RIN value decreases slightly to 8,0 and 8,1. 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).

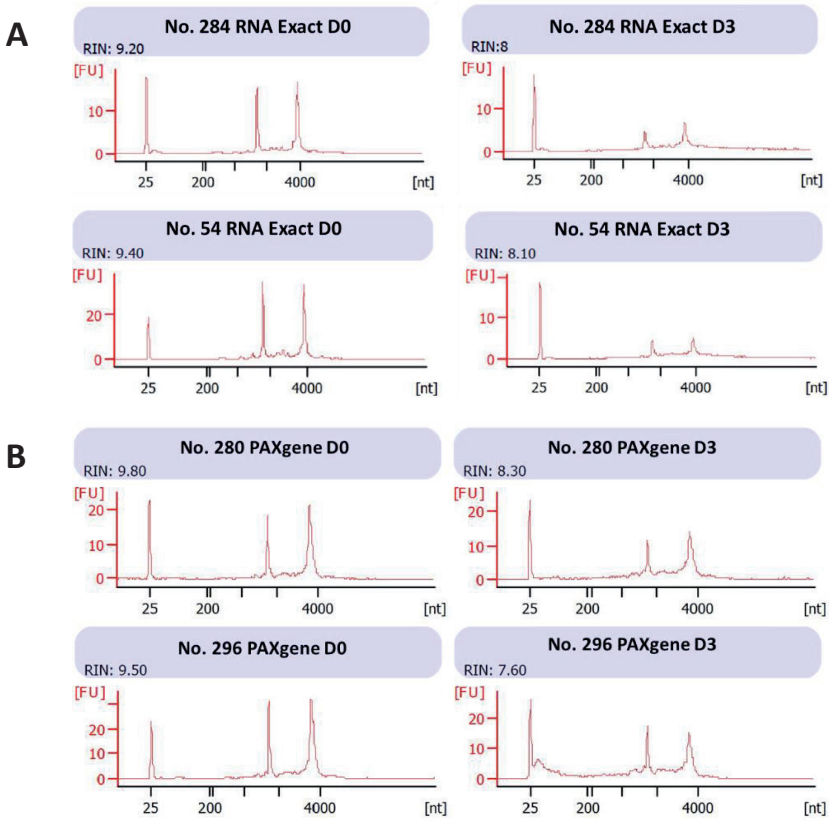

Figure 1: RNA integrity was measured with a Bioanalyzer 2100 (Agilent, Waldbronn, Germany) using RNA Nano 6000 Chips (Agilent REF 5067-1511). Data is shown exemplary for two donors each. (A) Isolation from the S-Monovette® RNA Exact were performed according to the protocol appended and show RIN values of 9,2 and 9,4 for day 0 or 8,0 and 8,1 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. The C_T values of the different time points and systems are shown in Figure 2. The C_T values of 10 or 9 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 the S-Monovette® RNA Exact or from the PAXgene® Blood tubes, either directly at day 0 or after 3 days of storage at room temperature. The dotted lines show C_T values 15 and 25 for orientation purposes.
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.

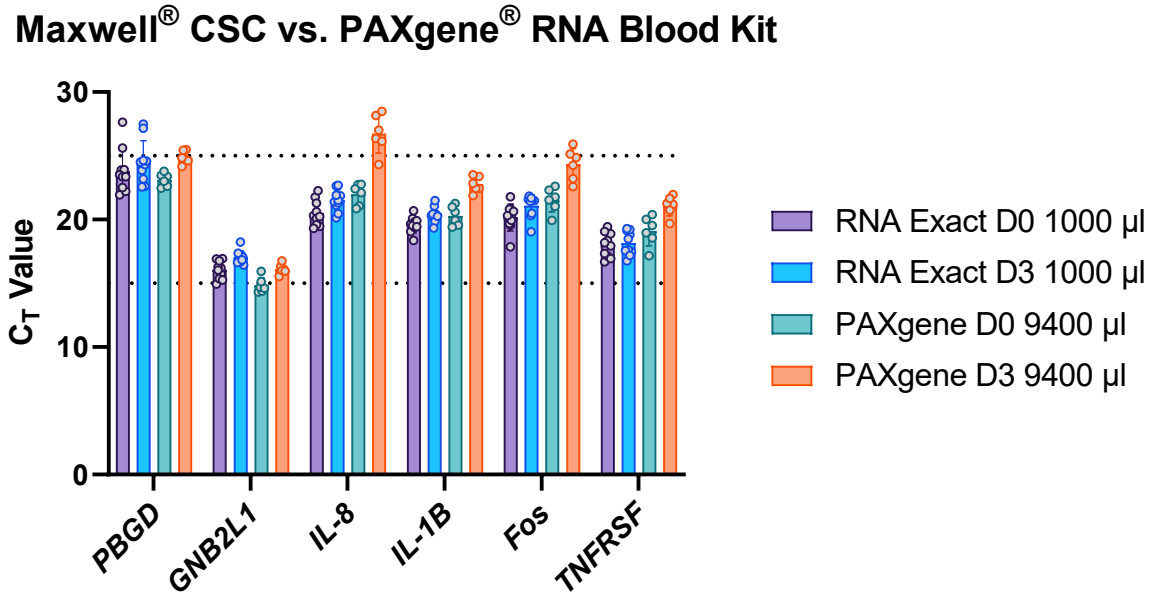

Figure 2: PCR Results after isolation with Maxwell® CSC RNA Blood Kit (REF AS1410). The C_T values of 10 or 9 independent donors were plotted here. The mean values and standard deviations are shown. Two housekeeping genes and four other genes were evaluated and the results were analyzed after RNA isolation from the S-Monovette® RNA Exact or from the PAXgene® Blood tubes, either directly at day 0 or after 3 days of storage at room temperature. The dotted lines show 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.

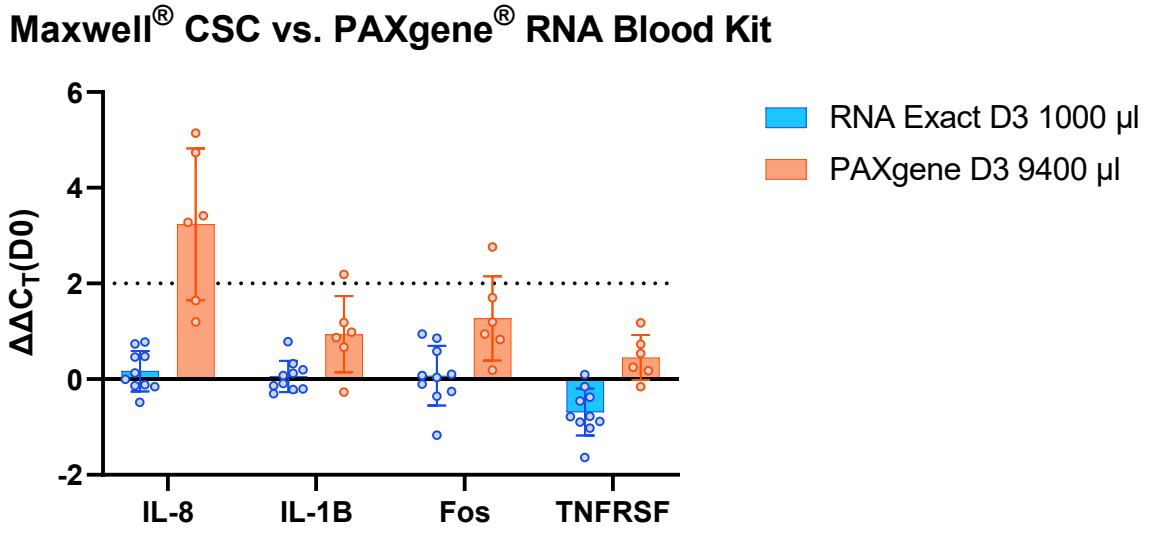

Figure 3: Calculation of the $\Delta\Delta C_T$ values for 10 or 9 different donors compared to the Day 0 samples. Depicted are the single values and the mean values with the standard deviation for 4 genes. The dotted line shows $\Delta\Delta C_T$ value 2 for orientation.

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. Dependent on the miRNA species the C_T values obtained with the Maxwell/RNA Exact system are comparable or minimal higher on fresh samples (D0) compared to the corresponding PAXgene® samples. But in contrast to the PAXgene® samples, the C_T values keep stable after storing the blood collection tube for three days at 25 °C.

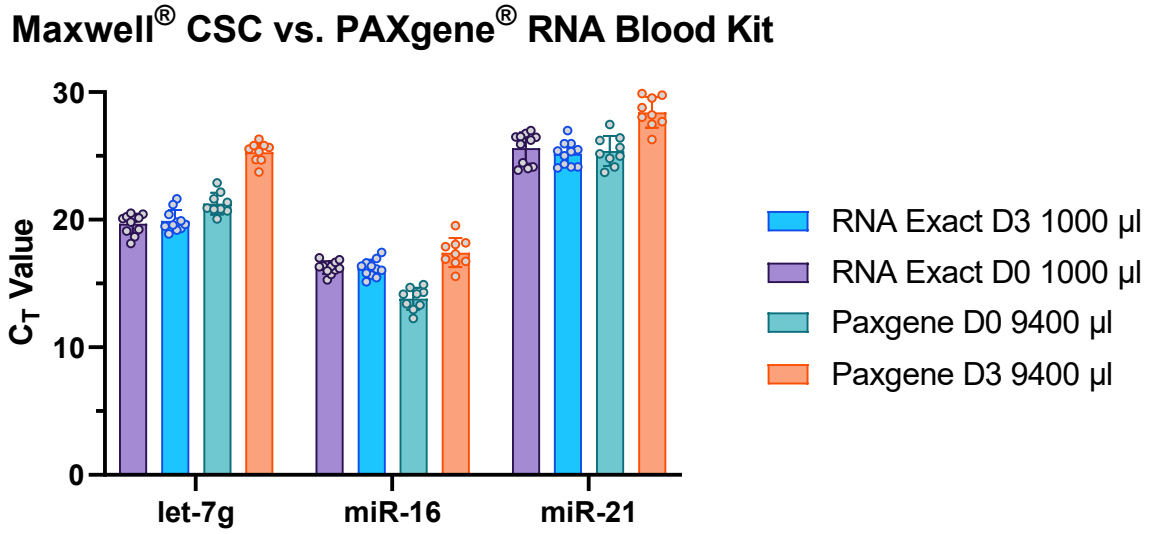

Figure 4: PCR Results for three miRNAs after isolation with Maxwell® CSC RNA Blood Kit or the PAXgene® Blood miRNA Kit. The C_T values of 10 or 9 independent donors are plotted here. The mean values and standard deviations are shown. Three different micro RNAs were evaluated.

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).

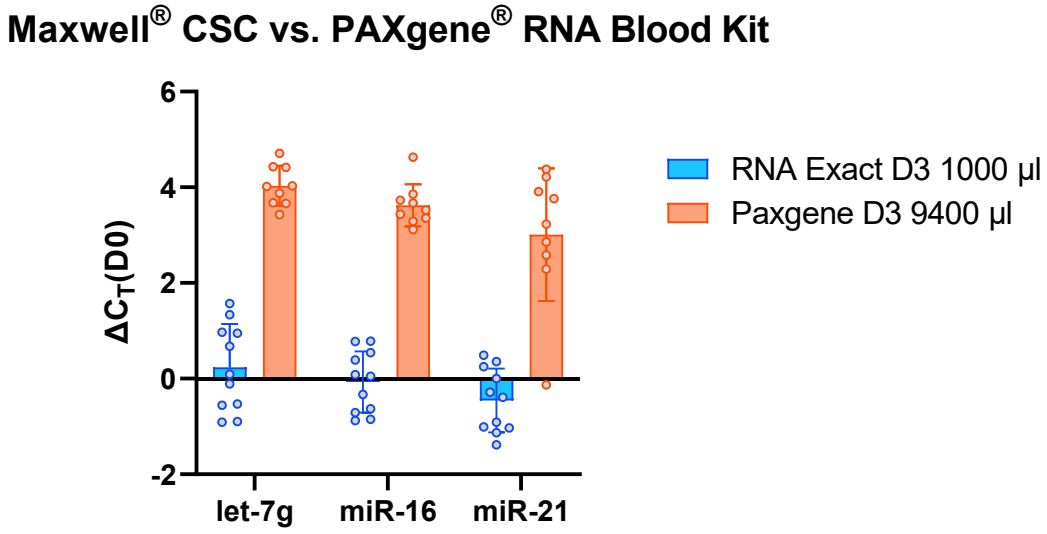

Figure 5: Calculation of the delta C_T values compared to Day 0 from the S-Monovette® RNA Exact or the PAXgene® blood Tube. Either 10 or 9 independent donors are plotted. Depicted are the single values and the mean values with the standard deviation for 3 miRNAs.

Summary

In this application note we show the compatibility of S-Monovette® RNA Exact stabilized samples with Maxwell® CSC RNA Blood Kit (REF AS1410) from Promega Corporation. With the RNA Isolation Kit, originally developed for whole blood from EDTA tubes, 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.

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 the sample preparation is reduced to a minimum. No centrifugation or additional preparation of solutions is required. The lysis carried out in the S-Monovette allows the sample to be transferred directly into the cartridge. This also significantly simplifies the standard protocol of the Maxwell® CSC RNA Blood Kit when blood is processed (Figure 6).

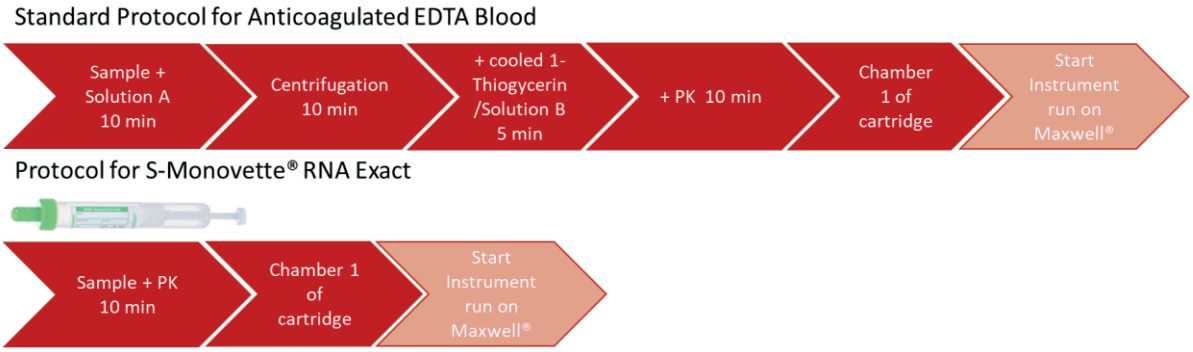

Figure 6: The figure shows the time saved when using the S-Monovette® RNA Exact instead of the standard sample input like anticoagulated EDTA blood.

The isolation efficiency of the combination of Maxwell® CSC RNA Blood Kit (REF AS1410) 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 without the entire sample quantity having been processed due to the volume limited cartridge system.

Dependent on the individual needs the user has the possibility for upscaling by using replicates, additionally there is enough to freeze a reserve sample.

DETAILED ISOLATION PROTOCOL

1. Proteinase K digestion of S-Monovette® RNA Exact samples

- Take 1,000 µl sample from S-Monovette® RNA Exact and incubate it in a 1,5 ml tube (e.g. REF 72.706.200) with 25 µl Proteinase K for 10 minutes at 25 °C, while shaking it in a thermomixer at 1,000 rpm.

During incubation prepare Maxwell® CSC RNA Blood Cartridges as described in Paragraph 2.

2. Prepare Maxwell® CSC RNA Blood Cartridges

- **Completely empty chamber 1 (the largest chamber in the cartridge) of the Maxwell® CSC RNA Blood Cartridges (the containing lysis-binding-buffer is not needed)**
- Place a CSC/RSC plunger in chamber 8 of each cartridge.
- Place an empty elution tube at the designated position for each cartridge in the cartridge holder(s).
- Add 50 µl of nuclease-free water to each elution tube. The elution tubes must remain open during RNA purification.

Note: Use only the nuclease-free water included in the Maxwell® CSC RNA Blood Kit. The use of other elution buffer solutions may affect RNA purification performance or subsequent use.

- Add 10 µl of reconstituted DNase I (blue) to chamber 4 (yellow) of each cartridge. The resulting green color is a visual indicator that the DNase I solution has been added to chamber 4.

3. Add S-Monovette® RNA Exact samples

- Add lysate to the empty chamber 1 of the Maxwell® CSC RNA Blood Cartridge (the largest chamber in the cartridge).

4. Instrument Run

- The following steps for the device run are carried out as described in the manufacturer‘s protocol.

Cap and remove the Elution Tubes containing RNA immediately following the run to prevent evaporation of the eluates. The purified samples are ready for immediate use. RNA samples may be stored overnight at -30 °C to -10 °C, or at lower than -60 °C for longer term storage.

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