Simultaneous extraction of mRNA and microRNA with the Norgen Total RNA Purification Kit Dx (REF Dx17200) from S-Monovette® RNA Exact

Schrage K.[1] , Schulz D.[2], Hehlert J.[1], Kämper M.[1]

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

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).
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 manual isolation with the Total RNA Purification Kit Dx (REF Dx17200) from Norgen Biotek Corporation.
The widely used PAXgene® Blood RNA System comprises one of the described RNA stabilizing tubes, which is why we use this as a comparison system to our S-Monovette® RNA Exact with the kit described. The system consists of a blood collection tube (PAXgene® Blood RNA Tube) and different RNA isolation kits, including a miRNA purification kit (PAXgene® Blood miRNA Kit).

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 also into PAXgene® Blood RNA Tubes (REF 762165, PreAnalytiX GmbH, Hombrechtikon, Switzerland).
PAXgene® Blood RNA Tubes 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 directly 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) with the PAXgene® Blood miRNA Kit (REF 763134, PreAnalytiX GmbH, Hombrechtikon, Switzerland). From S-Monovette® RNA Exact 600 µl lysed Blood at different time points (D0 and D3) was used for isolation with the Total RNA Purification Kit Dx (REF Dx17200Norgen Biotek Corporation, Thorold, Canada) . Isolation was performed according to an adapted 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. A detailed protocol for RNA isolation from S-Monovette® RNA-Exact is 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 mRNA was reversely transcribed into cDNA using the first strand cDNA Synthesis Kit (#K1612, Life Technologies, Darmstadt, Germany) according to manufacturers' instructions.

To investigate mRNA 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 Total RNA Purification Kit Dx from S-Monovette® RNA Exact.

gene	Accession No.	primer	Amplicon length
Porphobilinogen deaminase (PBGD)	NM_000190.33	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 for reverse Transcription (REF4366596, Applied Biosystems™, Waltham USA) 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 the manufacturer's instructions.

Results & Discussion

The samples were separated by capillary electrophoresis to visualize the RNA integrity. In most cases, the RNAs isolated with the PAXgene® system achieve RIN values above 9 at test time D0. The RIN values drop to around 7 after 3 days of sample storage. The signals from the isolates from the S-Monovette® RNA Exact are generally weaker, the RIN values at D0 are between 8 and 9 and drop to values between 7 and 8 after three days of storage. (Figure 1).

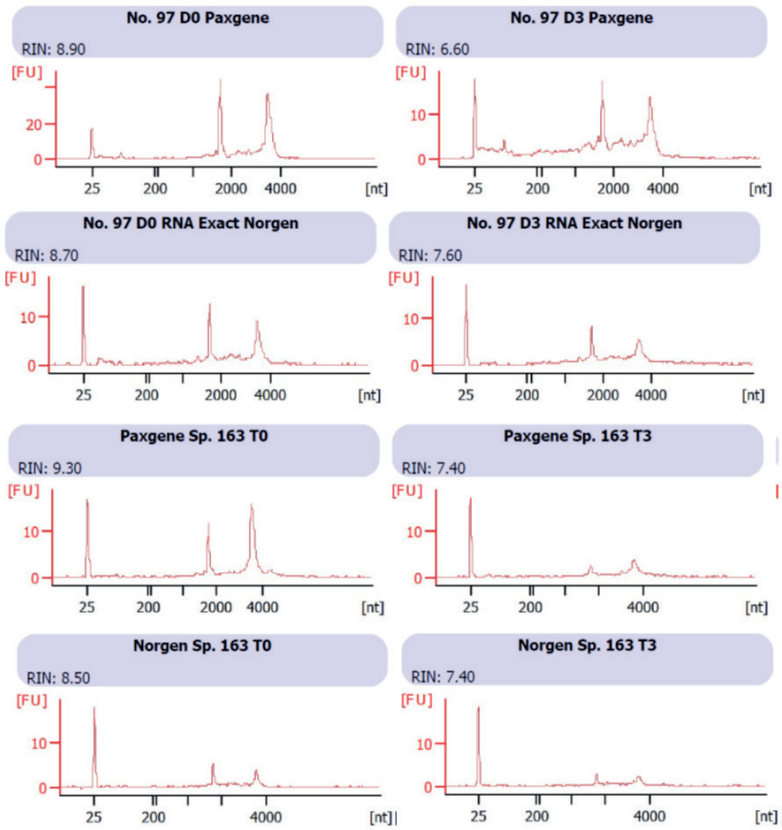

Figure 1: RNA integrity was measured with a Bioanalyzer 2100 (Agilent, Waldbronn, Germany) using RNA Nano 6000 Chips (Agilent REF 5067-1511). Data is shown exemplary for two donors each. The PAXgene® samples show RIN values of 8,9 and 9,3 for day 0 and 6,6 and 7,6 for day 3. Isolation from the S-Monovette®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

mRNA Analysis

Reverse transcription PCR and real-time PCRs of the isolated samples with four different genes and two housekeeping genes were performed. The CT values of the different time points and systems are shown in Figure 2. The results from the S-Monovette® RNA Exact show higher CT values, but with an outcome of a 10-fold lower sample volume (arithmetically around 3 CT values difference) it is obvious that the isolation efficiency of the Norgen Total RNA Kit in combination with the S-Monovette® RNA Exact is strongly superior compared to the PAXgene® system. In addition, the signals from the samples stored for 3 days correspond more closely to those from the fresh frozen samples, which indicates better stabilization of the RNA (Figure 2).

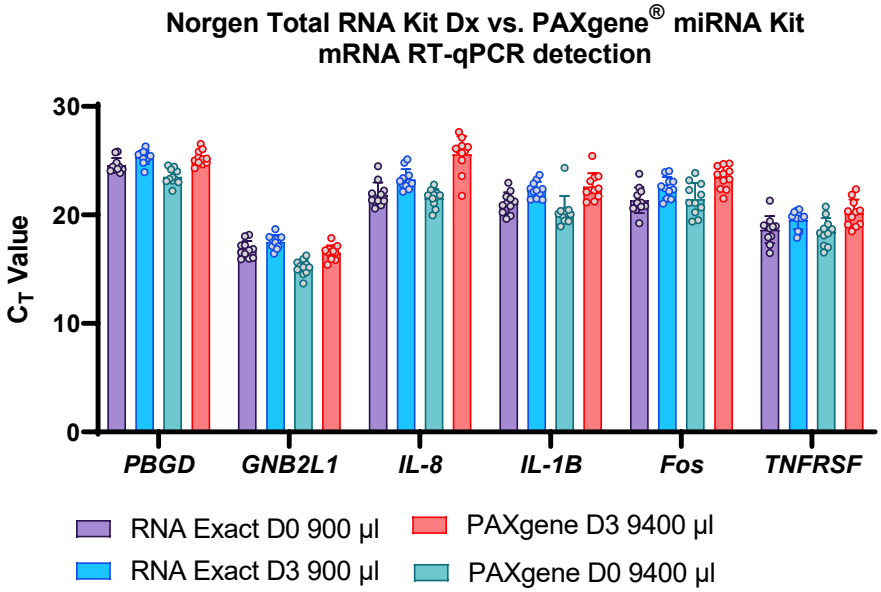

Figure 2: PCR results after isolation with Total RNA Purification Kit Dx. The CT values of 11 independent donors were plotted here. The mean values and standard deviations are shown. Two housekeeping genes and four other genes were evaluated and the results were analyzed after RNA isolation from PAXgene® Blood tubes or from the S-Monovette® RNA Exact, either directly at day 0 or after 3 days of storage at room temperature.

Figure 3 shows the ΔΔCT values calculated to the CT value from the day 0 samples of both systems after correction of the isolation efficiency via the CT-values of the housekeeping genes. This type of presentation highlights that the transcripts of the four selected genes are better stabilized in the S-Monovette® RNA Exact than in the PAXgene® blood collection tube (Figure 3).

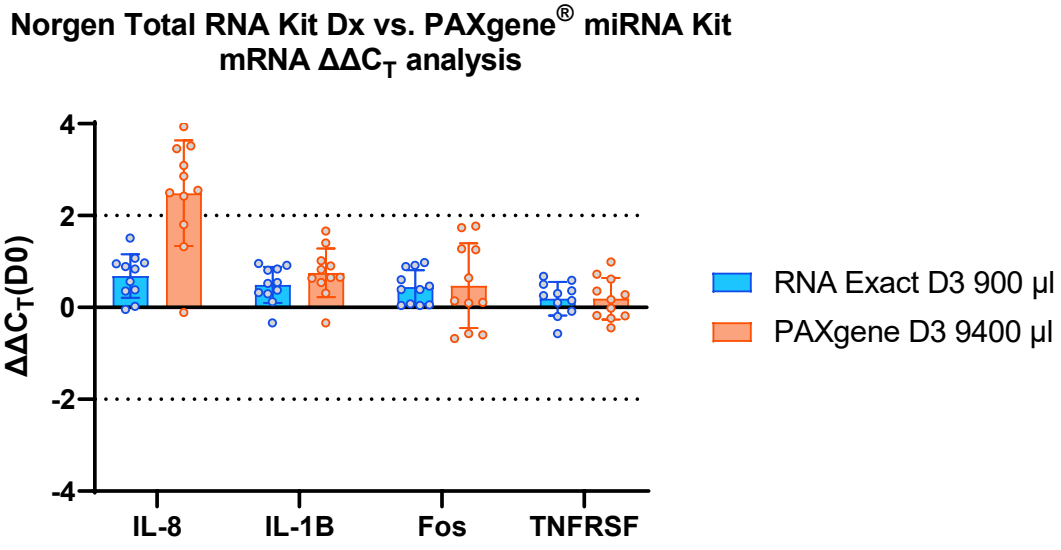

Figure 3: Calculation of the $\Delta\Delta C_T$ values for 9 different donors compared to the Day 0 samples. Depicted are the single values and the mean values with the standard deviation for 4 genes. The dotted lines show $\Delta\Delta C_T$ values -2 and 2 for orientation.

miRNA Analysis

Additional to mRNA analysis we also tested for micro RNA isolation by analyzing three different micro RNAs (miRNAs) in the S-Monovette® RNA Exact samples compared to the PAXgene® system. Despite the significantly lower sample volume the CT-values of the selected miRNAs obtained with the combination of Norgen/RNA Exact on fresh samples (D0) are remarkably lower compared to the corresponding PAXgene® samples and additionally in contrast to these samples the CT values keep much more stable after storing the blood collection tubes for three days (Figure 4).

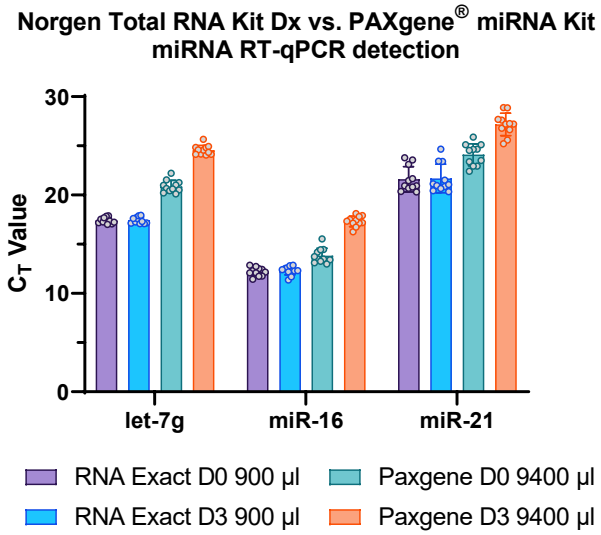

Figure 4: PCR Results for three miRNAs after isolation with Total RNA Purification Kit Dx or the PAXgene® Blood miRNA Kit. The CT values of 11 independent donors are plotted here. The mean values and standard deviations are shown. Three different micro RNAs were evaluated and the results were analyzed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

After calculating the Δ of the CT-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 ΔCT 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).

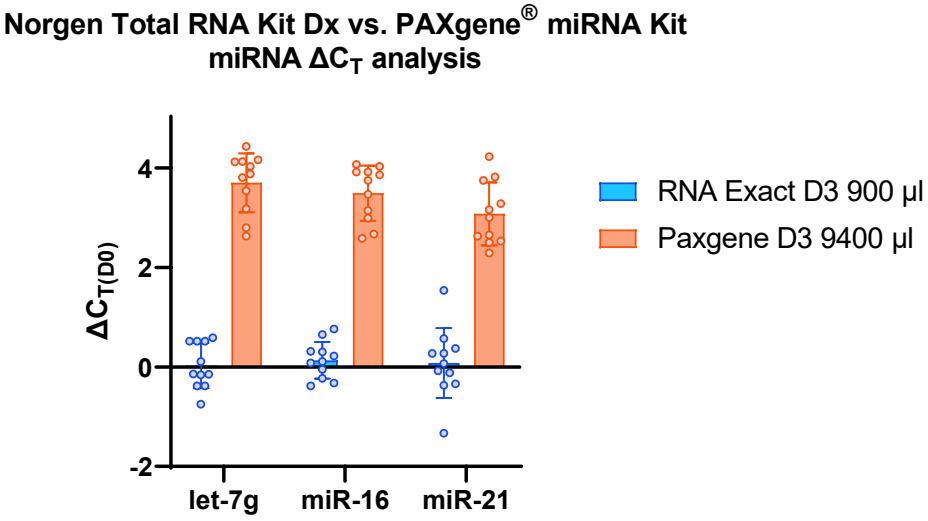

Figure 5: Calculation of the delta CT values compared to Day 0 from the S-Monovette® RNA Exact or the PAXgene® blood Tube. 9 independent donors are plotted. Depicted are the single values and the mean values with the standard deviation for 3 miRNAs.

Summary

In this application note we show the compatibility of S-Monovette® RNA Exact stabilized samples with Total RNA Purification Kit Dx (REF Dx17200) from Norgen Biotek Corporation. With the RNA Isolation Kit, originally developed for a variety of input samples, high quality mRNA and miRNA could be isolated using lysed blood generated from S-Monovette® RNA Exact.
The combination of S-Monovette® RNA Exact and Total RNA Purification Kit Dx is particularly suitable for the isolation of mRNA and microRNA. In the area of microRNA, RT-PCR signals can be obtained from comparatively small sample quantities that far exceed those from established systems such as PAXgene®. Taking into account the earlier detection and the smaller sample volume, the isolation efficiency of the Exact/Norgen system for miRNA is about 100x higher than that of the PAXgene® miRNA system.
Dependent on the individual needs the user even has the possibility for upscaling by isolation replicates and can additionally freeze a reserve sample.
Compared to the system from PAXgene®, considerable simplifications in the workflow are possible.
Manual work is reduced by at least half (Figure 6)!

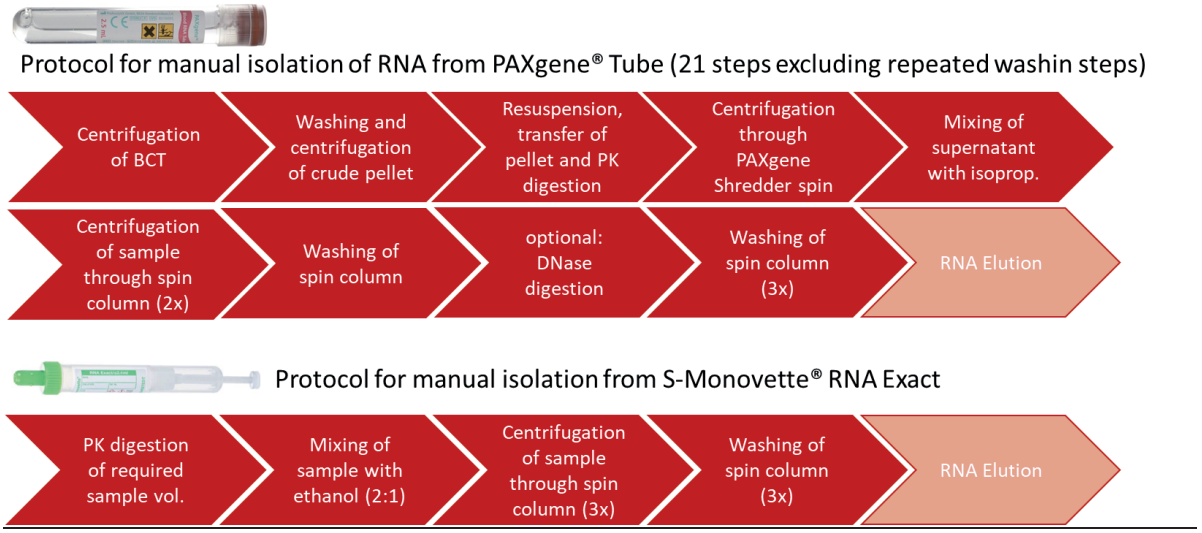

Figure 6: The figure illustrates the time saved when using the S-Monovette® RNA Exact instead of the PAXgene® miRNA isolation System

DETAILED ISOLATION PROTOCOL

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

- Transfer a sample volume of up to 900 µl (contains 225 µl blood) to a 2,0 ml reaction tube (e.g. Sarstedt REF.72.695.400).
  Add 15 µl Proteinase K (50 mg/ml), incubate 20 min at 25°C with 750-1000 rpm.

2. Adjust Binding conditions

- Add 450 µl ethanol 100 % to the 900 µl sample and mix.

3. Binding RNA to column

- Apply up to 600 µl onto the column and centrifuge for 1 minute (10000 – 13000 x g)
- Discard the flow through and reassemble or change collection tube
- (additional collection tubes e.g. Sarstedt REF. 72.708)
- Depending on your lysate volume, repeat this step as necessary

Note: Ensure the entire lysate volume has passed through into the collection tube by inspecting the column. If the entire lysate volume has not passed, spin for an additional minute.
Optional Step: if necessary for your downstream application carry out Norgen´s On-Column DNA Removal Protocol according to the description in the Kit´s protocol in Appendix A.

4. Column Wash

- Apply 400 µL of Wash Solution to the column and centrifuge for 1 minute.
- Discard the flow through and reassemble the spin column with its collection tube.
- Repeat the upper steps 2x to wash column a second and a third time.
- Discard the flow through and reassemble the spin column with its collection tube.
- Spin the column for 2 minutes with 13.000 x g in order to thoroughly dry the resin. Discard the collection tube.

5. RNA Elution

- Place the column into a fresh 1.5 mL Elution tube provided with the kit.
- Add 50 µL of Elution Solution to the column.
- Centrifuge for 2 minutes at 200 x g, followed by 1 minute at 13,000 x g.

6. Storage of RNA

- The purified RNA sample may be stored at –20°C for a few days.
- It is recommended that samples be placed at -80°C for long term storage.

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For further information please send us an e-mail to marketing@sarstedt.com or visit us at www.sarstedt.com.

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

www.sarstedt.com
info@sarstedt.com

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