MACHEREY-NAGEL – NucleoSpin Blood Kit (REF 740951.50) for Isolation of S-Monovette® cfDNA Exact stabilized samples

INTRODUCTION

The biomarker cell free DNA (cfDNA) is playing an increasingly important role in research and diagnostics. For blood sample stabilisation SARSTEDT offers the innovative S-Monovette® cfDNA Exact ensuring excellent sample quality with a guaranteed stabilisation performance for 14 days at 4 - 37°C (Schrage *et al.* 2022). Compatibility of blood collection tubes (containing cfDNA stabilizer solution) with isolation kits for cfDNA can be impaired due to the fact that cfDNA in the sample can be modified due to fixation by the stabiliser solution prefilled in the tubes. Therefore, we are pleased to show that S-Monovette® cfDNA Exact is compatible with a wide range of cfDNA isolation kits, and in particular with NucleoSpin Blood Kit from MACHEREY-NAGEL (REF 740951.50) as shown in the following application note. The details, and protocol adaptions, if necessary, are listed below.

MATERIAL & METHODS

Blood sample collection and storage:

Blood from four healthy donors was drawn into S-Monovette® cfDNA Exact blood collection tubes (REF 01.2040.001, SARSTEDT AG & Co. KG, Nümbrecht, Germany) or S-Monovette® K3 EDTA (REF. 01.1605.001, SARSTEDT AG & Co. KG, Nümbrecht, Germany).
Plasma was separated within one hour after blood collection (D0) or after storage of the blood collection tube for 3 days at 25 °C (D3) by two step centrifugation as described in the cfDNA Exact Monovette´s manual or the protocol below. The plasma was stored at -80 °C until cfDNA isolation.

cfDNA isolation:

1 ml of plasma from S-Monovette® cfDNA Exact and S-Monovette® K3 EDTA each at different time points were lysed and cfDNA was isolated as described in the manufacturers manual taking into account the input dependent scaling of the buffer volumes. A detailed protocol is attached.

cfDNA analysis:

To assess the cfDNA quality 1 µl of the eluate was visualised electrophoretically on a High Sensitivity DNA Chip (Agilent REF 5067-4626) with the Bioanalyzer 2100. The applicability of the isolated cfDNA to be used in common analysis methods was shown by using the samples in a qPCR assay to amplify the single copy genes *myostatin* (*MSTN;* Breitbach *et al.*, 2014) and a human *endogenous retrovirus* (*ERV-3;* Devonshire *et al.*,2014). The qPCR reactions were run with 8 µl sample input using the Maxima SYBR Green/ROX qPCR Master Mix (REF K0223, Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to manufacturer's protocol on a Mastercycler ep realplex 4S (Eppendorf, Hamburg, Germany) or Real-time Thermal cycler qTOWER^3 (Analytic Jena GmbH, Jena, Germany). Primers (see Table 1) were used in a final concentration of 0.5 µM.

Table 1. Primer sequences

Primer	Sequence	Annealing temp.	Fragment length
ERV-3fw (Devonshire *et al.* 2014)	5'-CATGGGAAGCAAGGGAACTAATG-3'	60 °C	135 bp
ERV-3rev	5'-CCCAGCGAGCAATACAGAATTT-3'		
MSTNfw (Breitbach *et al.* 2014)	5'-TTGGCTCAAACAACCTGAATCC-3'	60 °C	88 bp
MSTNrev	5'-TCCTGGGAAGGTTACAGCAAG-3'		

RESULTS & DISCUSSION

Following the cfDNA isolation, quality control was carried out via an Bioanalyzer (Agilent) with the High Sensitive DNA Analysis Kit. Figure 1 shows the capillary electrophoretic separations of two donors. The stabilisation performance of the S-Monovette® cfDNA Exact becomes particularly clear here, since in contrast to storage of the S-Monovette® K3 EDTA, no release of genomic DNA can be detected on day 3. For the donors shown, the cfDNA peak has a low level, but this is not surprising as the donors are a healthy control group.

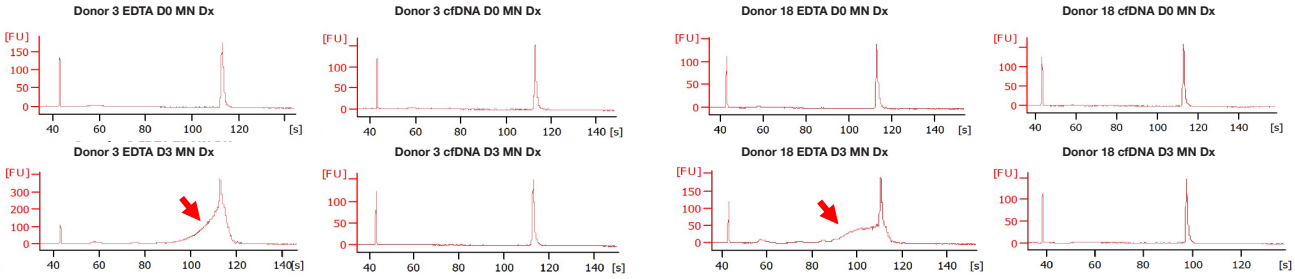

Figure 1: Capillary electrophoretic representation of cfDNA using a Bioanalyzer (Agilent). Illustration of two donors at the time point day 0 and after 3 days at 25 °C. The red arrow shows an entry of genomic DNA on day 3 into the sample from the S-Monovette® K3 EDTA (not stabilized samples); which is absent in the S-Monovette® cfDNA Exact due to stabilisation.

Subsequently, real-time PCRs of the isolated samples from 4 donors with two single-copy genes were performed. The C_T values of the 4 time points are shown in figure 2a. Figure 2b shows the ΔC_T values calculated to the C_T value from the day 0 samples of the S-Monovette® K3 EDTA which was processed immediately after blood collection (gold standard). Here, both the good isolation efficiency from the S-Monovette® cfDNA Exact and the stabilisation performance on day 3 at 25 °C become clear. No deviations beyond a donor-dependent variation could be detected.

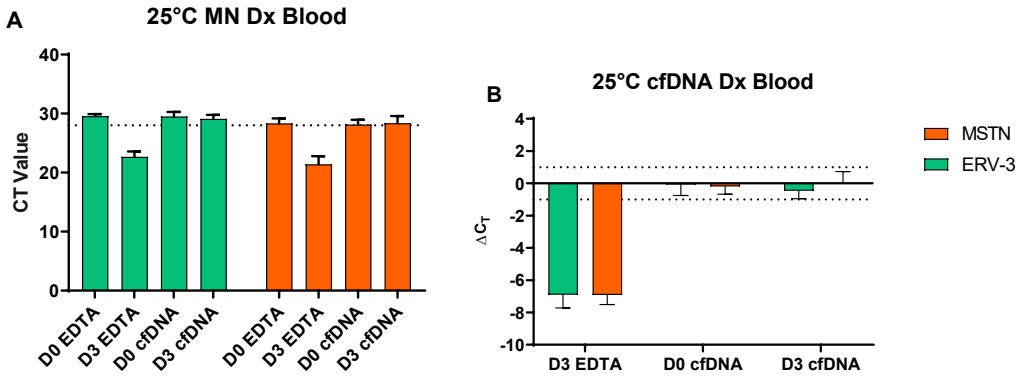

Figure 2: (A) C_T values from the tested single copy genes are shown at different time points. Average values from 4 donors with standard deviations were calculated. (B) Delta C_T value calculation for comparison to EDTA at time 0 were carried out. Values are shown averaged from 4 donors for two single copy genes with standard deviations.

SUMMARY

In this application note we show the compatibility of S-Monovette® cfDNA Exact stabilized samples with NucleoSpin Blood Kit from MACHEREY-NAGEL. With NucleoSpin Blood Kit, originally developed for gDNA isolation from whole blood samples high quality cfDNA could be isolated using plasma generated from S-Monovette® cfDNA Exact. Protocol adaptions are only necessary due to upscaling of the sample volumes which are necessary because of the lower cfDNA content of plasma compared to gDNA in a whole blood sample. The isolation of a sample volume larger than 1 ml plasma with the MACHEREY-NAGEL NucleoSpin® Blood kit is not recommended because of the limited volume of the column. For isolation of cfDNA from larger plasma volumes you may use the MACHEREY-NAGEL NucleoSnap cfDNA Kit (REF 740300.50) or one of the verified bead based extraction systems. Additionally, it is shown that sample stabilisation of cfDNA samples is indispensable to achieve good sample quality.

DETAILED ISOLATION PROTOCOL

Prepare cell-free plasma samples from S-Monovette® cfDNA Exact

1. **Replace the red screw-on lid with the centrifugation cap included**
2. Centrifuge the blood samples at 2.000-3.000 x g for 10 minutes at room temperature
3. Transfer the plasma into a new centrifugation tube (e.g. SARSTEDT REF 72.701.400 or 62.554.100)
4. Centrifuge the plasma samples at 15.000 x g for 15 minutes at room temperature and transfer supernatant into on or several new tubes. Continue to isolate cfDNA or store the plasma at -80 °C until further use.

Scaling table for sample preparation

Plasma volume [µl]	Proteinase K [µl]	Buffer B3 [µl]	Ethanol [µl]
200	25	200	210
400	25	400	420
1.000	25	1.000	1.050

Volumes of wash buffers and Proteinase K have not to be scaled, elution can occur in a volume of 50 µl elution buffer independent of the sample input volume.

Lyse plasma samples

1. Submit **1.000 µl Plasma** to a lysis tube with suitable volume (e.g. 5 ml reaction tube SARSTEDT REF 72.701.400)
2. Add 25 µl Proteinase K
3. Add **1.000 µl B3**, mix
4. Incubate 5 min at room temperature
5. Place tube(s) into an Eppendorf ThermoMixer® heat up to 70 °C with tubes placed inside, than incubate for 10 minutes while mixing (1.000 rpm)
6. Spin down to collect the fluid

Adjust binding conditions

7. Add **1.050 µl Ethanol** (98-100 %) and mix
8. Spin down to collect the fluid

Bind DNA to column matrix, repeat this step until all lysate passed the column

9. Load up to 700 µl lysate
10. Centrifuge 11.000 x g, 1 min
11. Transfer the column to a new collection tube
12. Repeat loading of columns until the plasma volume is processed

Wash silica membrane

13. Add 500 µl washing buffer BW
14. Centrifuge 11.000 x g, 1 min
15. Transfer the spin column to a new collection tube
16. Add 600 µl B5 desalting buffer
17. Centrifuge 11.000 x g, 1 min

Dry silica membrane

18. Transfer the spin column to a new collection tube
19. Centrifuge 11.000 x g, 1 min

Elute cfDNA

20. Transfer the spin column to an elution tube
21. Add 50 µl elution buffer BE onto the centre of the membrane
22. Centrifuge 11.000 x g, 1 min
23. Discard the column and store the eluted cfDNA at -20 °C or below until further analysis

Publication bibliography

Breitbach, Sarah; Tug, Suzan; Helmig, Susanne; Zahn, Daniela; Kubiak, Thomas; Michal, Matthias *et al.* (2014): Direct quantification of cell-free, circulating DNA from unpurified plasma. In *PloS one* 9 (3), e87838. DOI: 10.1371/journal.pone.0087838.

Devonshire, Alison S.; Whale, Alexandra S.; Gutteridge, Alice; Jones, Gerwyn; Cowen, Simon; Foy, Carole A.; Huggett, Jim F. (2014): Towards standardisation of cell-free DNA measurement in plasma. Controls for extraction efficiency, fragment size bias and quantification. *In Analytical and bioanalytical chemistry* 406 (26), pp. 6499–6512. DOI: 10.1007/s00216-014-7835-3.

Schrage, Kathrin; Linden, Justus; Kämper, Martin; Reiter, Jenny; Schuster, Rainer (2022): Comparison of S-Monovette® cfDNA Exact with two blood collection tubes for stabilization of cfDNA. SARSTEDT AG & Co. KG. Nümbrecht. Available online at https://www.sarstedt.com/fileadmin/user_upload/Mediacenter/Studien/Studie_neu/an_009_comparison_of_smonovette_cfdna_exact_0922.pdf.

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