MACHEREY-NAGEL – NucleoMag® cfDNA kit (REF 744550.4) 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 NucleoMag cfDNA kit from MACHEREY-NAGEL (REF 744550.4) as shown in the following application note.

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., Nümbrecht, Germany) or S-Monovette® K3 EDTA (REF 01.1605.001, Sarstedt AG & Co., 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:

1ml 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 can be found below.

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.

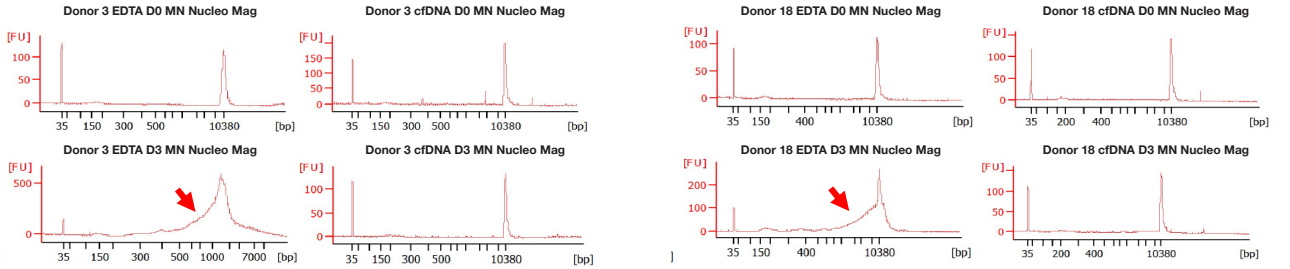

Figure 1: Capillary electrophoretic representation of cfDNA using a Bioanalyzer (Agilent). Illustration of two donors at the time point day 0 and after 3 days at 25°C. The red arrow shows an entry of genomic DNA on day 3 into the sample from the S-Monovette® K3 EDTA (not stabilized samples); which is 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 S-Monovette® cfDNA Exact stabilized samples and the stabilisation performance on day 3 at 25°C become clear. No deviations beyond a donor-dependent variation could be detected.

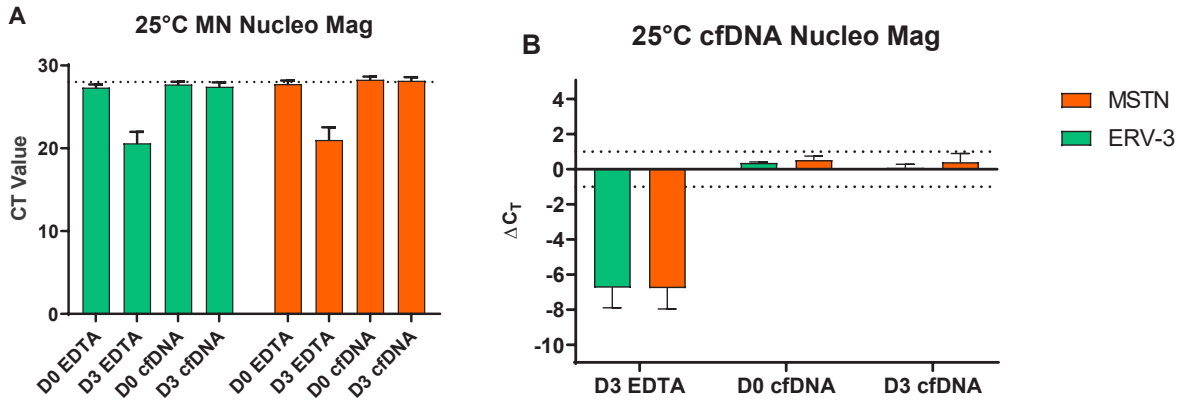

Figure 2: (A) C_T values from the tested single copy genes are shown at different time points. Average values from 4 donors with standard deviations were calculated. (B) Delta C_T value calculation for comparison to EDTA at time 0 were carried out. Values are shown averaged from 4 donors for two single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

SUMMARY

In this application note we show the compatibility of S-Monovette® cfDNA Exact stabilized samples with NucleoMag cfDNA kit from MACHEREY-NAGEL. With the kit, high quality cfDNA could be isolated following the instruction manual of the kits´ manufacturer without substantially adaptions. Small adaptions have been implemented to adapt the protocol for the use with single tubes. Additionally, it is shown that sample stabilisation of cfDNA samples is indispensable to achieve good sample quality.

Protocol for manual isolation of 1 ml plasma in single tubes

Preparation of 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
4. Centrifuge the plasma samples at 15.000 x g for 15 minutes at room temperature and transfer supernatant into a new tube. Continue to isolate cfDNA or store the plasma at -80°C until further use.

Scaling table for sample preparation

Plasma [µl]*	Prot. K [µl]	MCF1 [µl]	MCF2[µl]	Beads [µl]	MCF3/4 [µl]	MCF5 [µl]
1.000	25	450	1.000	15	500	50
2.000	50	900	2.000	30	1.000	75
4.000	100	1.800	4.000	60	1.000	100

*For other sample volumes please scale according to the table in the kit´s manual

Sample Lysis (please scale components depending of input volume as indicated in the manufacturers manual)

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. Incubate 15 min at room temperature preferably with shaking at 1.000 rpm
4. Add 450 µl MCF1, mix
5. Place tube(s) into an Eppendorf ThermoMixer® heat up to 56°C with tubes placed inside, than incubate for 30 minutes while mixing (1.000 rpm)
6. Spin down to collect the fluid

Bind the cfDNA to the beads

7. Add 1.000 µl MCF2 Buffer and mix
8. Add 15 µl NucleoMag® P-Beads and mix thoroughly
9. Shake vigorously for 10 minutes (for example on an Eppendorf™ ThermoMixer™ or Vortex tube adapter)
10. Place the tube on the appropriate magnet for 5 minutes (a short spin down of the liquid may be useful to collect all sample in the tube)
11. Carefully discard the supernatant with a pipette (remove residual supernatant after another minute on the magnet)

Wash the bound cfDNA

12. Remove the tubes from the magnet and resuspend the beads in 0,5 ml wash Buffer MCF3.
13. Transfer the beads slurry completely to a new 1.5 ml microcentrifuge tube (e.g. Sarstedt 72.704.400 or 72.704.200).
14. Place the microcentrifuge tube with the beads on an appropriate magnet until the solution is clear and collect the supernatant (you may use this supernatant to collect residual beads out of the Lysis/Binding Tube and combine them in the 1.5 ml microcentrifuge tube).
15. Remove all residual supernatant.
16. Remove the tube from the magnet, add 0,5 ml of wash Buffer MCF4, then vortex for 30 seconds.
17. Place the tube on the magnet (collecting the liquid via short centrifugation may be useful) until the beads are pelleted against the magnet, remove all supernatant.
18. Repeat the washing steps with wash buffer MCF4.
19. Remove all residual liquid and air dry the beads for 15 minutes.

Elute the cfDNA

20. Add 50 µl Elution buffer MCF5 and vortex until all beads are resuspended.
21. Incubate for 5 min at 56°C in an suitable Thermo Mixer with 1.000 rpm.
22. Centrifuge shortly, than pellet the beads to the magnet and save the clear supernatant in a new DNase free tube.
23. 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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