Applied Biosystems – MagMax™ Cell-Free DNA Isolation Kit (REF A29319) 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, in particular MagMax™ Cell-Free DNA Isolation Kit from with Applied Biosystems (REF A29319) as shown in the following application note. The details, and protocol optimisations, 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:

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 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
MSTNrevc	5'-TCCTGGGAAGGTTACAGCAAG-3'		

RESULTS & DISCUSSION

Following the cfDNA isolation, quality control was carried out via a Bioanalyzer 2100 (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.

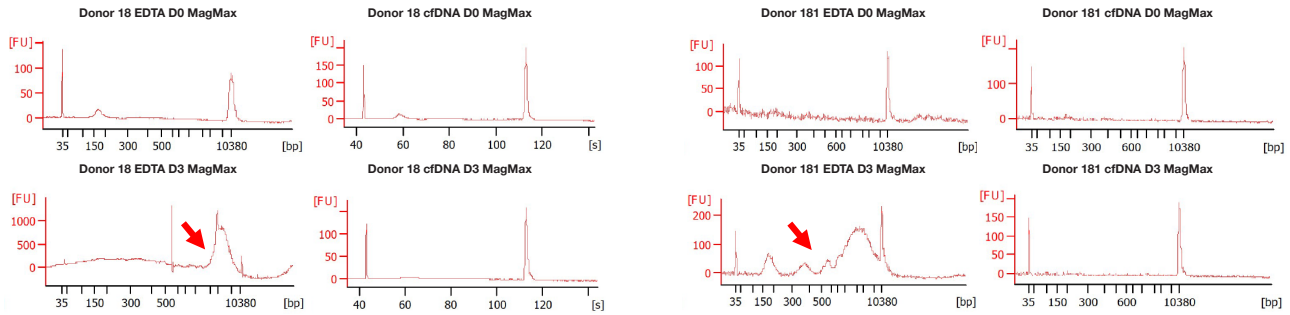

Figure 1: Capillary electrophoretic representation of cfDNA using a Bioanalyzer (Agilent). Illustration of two donors at the time point day 0 and after 3 days at 25 °C. The red arrow shows an entry of genomic DNA on day 3 into the sample from the S-Monovette® K3 EDTA (not stabilized samples); which is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

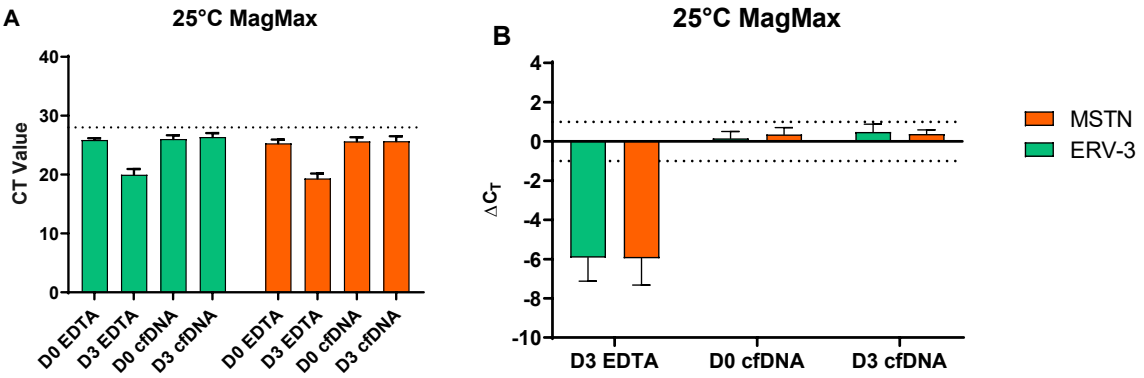

Figure 2: (A) C_T values from the tested single copy genes are shown at different time points. Average values from 4 donors with standard deviations were calculated. (B) Delta C_T value calculation for comparison to EDTA at time 0 were carried out. Values are shown averaged from 4 donors for two single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

SUMMARY

In this application note we show the compatibility of S-Monovette® cfDNA Exact stabilized samples with MagMax™ Cell-Free DNA Isolation Kit from with Applied Biosystems. With the kit, high quality cfDNA could be isolated following the manufacturers protocol with Proteinase K digestion. For automatic isolation, the protocol on the ThermoFisher website can be used. Small protocol adjustments which help to support the manual isolation are implemented in the protocol below. Additionally, it is shown that sample stabilisation of cfDNA samples is indispensable to achieve good sample quality.

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 2000-3000 x g for 10 minutes at room temperature
3. Transfer the plasma into a new centrifugation tube (for example 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
   (Alternatively, the plasma samples can be centrifuged at 6000 x g for 30 minutes to remove any residual debris)

Lyse plasma samples with Proteinase K (PK)

1. Add the following components to a tube in the order indicated

Reagents	Plasma volume		
	1 ml	2 ml	4 ml
Proteinase K, 20 mg/ml	15 µl	30 µl	60 µl
Plasma sample	1 ml	2 ml	4 ml
SDS, 10 % solution[1]	100 µl	200 µl	400 µl
Total volume	1.115 ml	2.230 ml	4.460 ml

[1] using a 20 % SDS solution as indicated in the original protocol is not recommended because of insufficient solubility

2. Mix well and incubate at 60 °C for 20 minutes on an Eppendorf™ ThermoMixer™ while shaking with 1000 rpm
3. Cool down the samples on ice for 5 minutes

Bind the cfDNA to the beads

1. Prepare the Binding Solution/Beads Mix according to the table and mix well

Reagents	Plasma volume		
	1 ml	2 ml	4 ml
MagMAX™ Lysis/Binding Solution	1.25 ml	2,5 ml	5 ml
MagMAX™ Magnetic Beads	15 µl	30 µl	60 µl
Total Volume	1.265 ml	2.53 ml	5.06 ml

2. Add the prepared Binding Solution/Beads Mix to each sample an mix thoroughly
3. Shake vigorously for 10 minutes (for example on an Eppendorf™ ThermoMixer™ or Vortex tube adapter)
4. 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)
5. Carefully discard the supernatant with a pipette (remove residual supernatant after another minute on the magnet)

Wash with Wash Solution and 80 % ethanol

1. Resuspend the beads in 1 ml of MagMAX™ Cell Free Wash Solution
2. Transfer the beads slurry completely to a new 1.5 ml microcentrifuge tube
3. 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)
4. Remove all residual supernatant
5. Remove the tube from the magnet, add 1 ml of 80 % ethanol, then vortex for 30 seconds
6. 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
7. Repeat the washing steps with 80 % ethanol
8. Remove all residual liquid and air dry the beads for 5 minute

Elute the cfDNA

1. Add MagMAX™ Cell Free DNA Elution Solution to the tube according to the following table

Reagents	Plasma volume		
	1 ml	2 ml	4 ml
MagMAX™ Elution Solution	30 µl	40 µl	60 µl

2. Vortex until all the beads are resuspended than incubate for 5 minutes in an Eppendorf ThermoMixer® (37 °C, 1400 rpm). Centrifuge shortly than pellet the beads to the magnet and save the clear supernatant.

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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