Original Article

The Effects of Different Storage Conditions and Repeated Freeze/Thaw Cycles on the Concentration, Purity and Integrity of Genomic DNA

(gDNA / storage / freeze / thaw cycles / concentration / purity / integrity)

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Abstract. The crucial requirement of molecular genetic methods is high-quality input material. The key question is "how to preserve DNA during long-term storage." Biobanks are recommended to aliquot isolated DNA into provided volumes. The aim of this study was to analyse the effect of repeated freezing and thawing on the genomic DNA integrity, quality and concentration. The aliquoted DNA isolated from blood cells using the automatic MagNA system and manual salting out method underwent freeze/thaw cycles at different storage conditions (−20 °C, −80 °C and liquid nitrogen). The average initial concentrations were 270.6 ng/µl (salting out method) and 125.0 ng/µl (MagNA). All concentration deviations relative to the concentration after the first freeze/thaw cycle were less than 5 % for −20 °C and −80 °C cycling with both isolation methods. The average percentage differences of liquid nitrogen samples were higher, and the MagNA isolation method showed significant differences. There were no significant changes in the DNA purity or quality. The repeating freeze/thaw up to 100 cycles (through −20 °C and −80 °C, respectively) did not significantly influence the integrity, concentration, or purity of genomic DNA, suggesting that storage of samples in high-volume pools without multiple aliquoting is possible. Storage in a freezer seems to be the most suitable way of long-term DNA preservation, because liquid nitrogen storage leads to formation of DNA clumps.

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Abbreviations: EDTA – ethylenediaminetetraacetic acid, ISO – International Organization for Standardization, TE – Tris-EDTA buffer, UV – ultraviolet.

Introduction

With the advances in molecular genetic methods, high quality of the input material becomes the crucial requirement for many analyses. The quality of genomic DNA isolated from blood cells is determined by both isolation procedure and storage. The extraction of DNA from peripheral blood can be made either manually (e.g., phenol-chloroform method) or using a commercial kit in automated workstation (e.g., MagNa Pure Compact System or QIAcube); and both methods lead to excellent results in terms of quality and quantity (Köchl et al., 2005; Kalousova et al., 2017). The key question is "how to store DNA", since research projects routinely use DNA from biobanks for their analysis. Thus, biobanks face an important decision. On the one hand, isolated DNA can be aliquoted into provided volumes and stored at −80 °C, which is the recommended temperature for long-term storage. This is recommended by ISO 20186-2:2019 (ISO, 2019). On the other hand, one or two high-volume aliquots can be made and stored. In case of need, this aliquot is thawed and frozen again after the desired volume has been pipetted off. The first possibility increases costs (e.g., energy and storage space) and the second one might influence the integrity of DNA because of repeated freeze/thaw cycles. Therefore, we decided to analyse the effect of repeated freezing and thawing on the genomic DNA integrity, quality and concentration in our short-term study in order to optimize storage procedures given to the storage capacity of our biobank (http://biobanka.lf1.cuni.cz/). We have

compared two extraction methods routinely used in our laboratory, the manual salting out procedure and automated isolation.

Materials and Methods

The study was performed with the approval of the local Institutional Ethical Committee and was conducted in accordance with the Declaration of Helsinki. All volunteers have given informed consent with participation in the study.

Blood samples were collected from each of 10 healthy anonymized volunteers (5 men / 5 women, mean age 46 ± 11 years) into tubes containing ethylenediaminetetraacetic acid (EDTA).

To obtain blood cells, the collected blood samples were centrifuged at 3,000 rpm (radius = 86 mm) for 15 min and almost all plasma was removed. These samples were stored in a refrigerator (2–8 °C) until DNA extractions were performed. Two extraction methods commonly used in our laboratory were chosen for DNA extraction: the manual salting out procedure described by Miller et al. (1988) with slight modification (Kalousova et al., 2017); and automated isolation performed using a MagNA Pure Compact Nucleic Acid Isolation Kit I – Large Volume (Roche Diagnostics, Mannheim, Germany) with the MagNA Pure Compact System (Roche Diagnostics). The commercial kit was used according to the manufacturer's instructions. Samples extracted by the salting out protocol were diluted with Tris-EDTA (TE) buffer (Tris-HCl, 10 mM, pH = 7,6; EDTA, 1 mM) to the maximal DNA concentration 300 ng/μl. The extracted DNA was quantified using absorbance at 260 nm (NanoDrop™ 1000 Spectrophotometer, ThermoFisher Scientific, Waltham, MA) and purity was estimated by calculation of absorbance ratio 260/280 nm. All initial values were in the range 1.8–2.0, which is considered to represent pure DNA. The DNA samples were stored in a refrigerator (2–8 °C) before the freeze/thaw experiments.

DNA from each subject was aliquoted and treated according to the following protocols (A–D). Protocol A did not include any freeze/thaw cycles and these aliquots were stored at 4 °C throughout the experiment. They subsequently served as DNA integrity controls. Protocol B and protocol C cycled through −20 °C and −80 °C, respectively, while protocol D used liquid nitrogen freeze/thaw cycles. The thawing process lasted for at least 30 min at room temperature. Afterwards, samples were frozen to the desired temperature, which took at least 3 h. The number of freeze/thaw repetitions for a single aliquot was 1, 2, 3, 4, 5, 10, 20, 50, or 100 in protocols B–D.

The integrity of DNA was visualized under UV light (Gel Logic, Kodak, Rochester, New York) after TBE (10X) gel electrophoresis in 0.7% agarose gel stained with GelRed (Biotium, Fremont, CA). The running conditions were 50 V for 10 min, followed by 100 V for 10 min and finished by 150 V for 50 min (Cleaver Scientific, Rugby, UK). All gels were run at room temperature. Before the electrophoretic separation, the concentration of each aliquot was measured as described above. The final amount of DNA loaded on the gel was 50 ng of each aliquot. GeneRuler 1kb DNA Ladder (Fermentas, Waltham, MA) was used as a molecular marker.

For statistical analysis, we used software STATISTICA 12 (Statsoft CR s. r. o., Prague, Czech Republic), WOLFRAM MATHEMATICA 12.0 (Wolfram, Long Hanborough, UK), and SPSS 20.0 (SPSS Inc, Chicago, IL). Descriptive statistics of numerical variables are presented in the form of mean ± standard deviation, and $P < 0.05$ was considered as significant. Basic comparison was performed using Wilcoxon matched-pairs signed-ranks test. The systematic concentration changes and their dependence on the used isolation method and storage temperature, respectively, was tested using two-way repeated-measures ANOVA. Effect sizes of both changes and dependencies was evaluated using the η^2 coefficient and categorized as a small/medium/large effect size according to Cohen's conventions (Cohen, 1992). The coefficients of these changes, including confidence intervals, were quantified using MIXED model generalization.

Results

The salting out extraction method gives higher DNA yields (mean concentration 270.6 ± 25.1 ng/μl) than the automatic method using the MagNA instrument (mean concentration 125.0 ± 21.4 ng/μl), $P < 0.01$. The initial concentrations measured before sample aliquoting and the concentrations of aliquots after one freeze/thaw cycle were used as controls for statistical analyses. Samples extracted by the salting out method kept their concentrations through both protocols B and C, cycling at −20 °C and −80 °C, respectively. We noticed a very interesting trend in concentrations of these samples stored in liquid nitrogen. After one freeze/thaw cycle, there was a significant increase in concentrations ($P < 0.01$) followed by lower concentrations except for one value. Some concentration decreases were statistically significant ($P < 0.05$). Samples isolated using the MagNA system did not show any differences in concentration during −20 °C cycling. However, concentrations after the first freeze/thaw cycle increased ($P < 0.01$) in protocol C (−80 °C cycling). The following phases had slightly lower concentrations in comparison with one freeze/thaw cycle aliquots, except for 50 times frozen and thawed samples, where the decrease was more appreciable ($P < 0.01$). The liquid nitrogen protocol had a similar trend as samples obtained with the salting out method. Significantly higher concentrations were observed after one freeze/thaw cycle in comparison with initial values ($P < 0.01$) and subsequent decrease in later phases of the protocol ($P < 0.01$). As expected, concentrations of all control samples stored in the refrigerator (2–8 °C) significantly increased ($P < 0.01$). Although some concentration fluctuations were statistically significant between the first/hundredth and the zeroth/first

freeze/thaw cycle, respectively, all concentrations except for one relative to the concentration after the first freeze/thaw cycle differed in less than 5 % for −20 °C and −80 °C cycling using both isolation methods (one value was 11 %). The percentage differences of liquid nitrogen samples were on average higher, especially in later phases of MagNA aliquot cycling. See Table 1 and Fig. 1 for detailed summary of concentration changes during the freeze/thaw cycling. All results mentioned above were obtained using basic statistics and Wilcoxon matched-pairs signed-ranks test.

The two-way repeated-measures ANOVA model showed that the concentrations changed significantly during the cycles ($P = 0.022$, $\eta^2 = 0.109$, medium effect size). However, the cycle number strongly interacted with the storage temperature ($P < 0.001$, $\eta^2 = 0.339$, large effect size). The mixed model showed that only MagNA-isolated DNA stored in liquid nitrogen significantly changed during the cycling ($P = 0.009$) and the coefficient of change was small from the practical point of view: −0.274 ng/µl per cycle, $CI_{0.95}$ = (from −0.479 to −0.069). All concentration changes per cycle are summarized in Table 2.

We did not observe any significant changes in DNA purity. The average values of all measured series were in the range 1.8–2.0 (the 260/280 nm ratio). The subsequent agarose electrophoresis did not show any changes in DNA quality between the frozen/thawed aliquots and control samples stored in the refrigerator (2–8 °C) during the experiments, see Fig. 2.

Table 1. Concentration changes of DNA isolated by the salting out method and MagNA during freeze/thaw cycles. Concentrations (ng/µl) are expressed as mean ± standard deviation.

	Salting out method							
Cycles	**Refrigerator (2–8 °C)**	**Concentration deviation (relative to)**	**−20 °C**	**Concentration deviation (relative to)**	**−80 °C**	**Concentration deviation (relative to)**	**Liquid nitrogen**	**Concentration deviation (relative to)**
0 (c0)	270.6 ± 26.4		270.6 ± 26.4		276.3 ± 20.4[x]		270.6 ± 26.4	
1 (c1)			263.2 ± 29.6	−2.7 % (c0)	277.0 ± 23.3	+0.3 % (c0)	313.1 ± 27.6[££]	+15.7 % (c0)
2 (c2)			258.8 ± 24.8	−1.7 % (c1)	283.7 ± 36.6	+2.4 % (c1)	302.9 ± 29.1	−3.3 % (c1)
3 (c3)			273.8 ± 35.9	+4.0 % (c1)	277.8 ± 25.0	+0.3 % (c1)	305.5 ± 30.6	−2.4 % (c1)
4 (c4)			266.1 ± 32.1	+1.1 % (c1)	270.1 ± 33.1	−2.5 % (c1)	315.9 ± 42.2	+0.9 % (c1)
5 (c5)			262.7 ± 26.0	−0.2 % (c1)	268.4 ± 20.1	−3.1 % (c1)	297.6 ± 31.0[*]	−5.0 % (c1)
10 (c10)			292.8 ± 61.7	+11.2 % (c1)	268.6 ± 19.9	−3.0 % (c1)	295.4 ± 28.0[##]	−5.7 % (c1)
20 (c20)			264.8 ± 27.7	+0.6 % (c1)	269.7 ± 22.6	−2.6 % (c1)	307.0 ± 37.0[*]	−1.9 % (c1)
50 (c50)			270.1 ± 29.3	+2.6 % (c1)	274.0 ± 22.7	−1.1 % (c1)	289.1 ± 38.1[*]	−7.7 % (c1)
100 (c100)			276.0 ± 40.1	+4.9 % (c1)	275.4 ± 21.9	−0.6 % (c1)	304.3 ± 37.9[*]	−2.8 % (c1)
After 3 months	339,9 ± 50,5[££]	+25.6 % (c0)						

	MagNA							
Cycles	**Refrigerator (2–8 °C)**	**Concentration deviation (relative to)**	**−20 °C**	**Concentration deviation (relative to)**	**−80 °C**	**Concentration deviation (relative to)**	**Liquid nitrogen**	**Concentration deviation (relative to)**
0 (c0)	125.0 ± 22.6		125.0 ± 22.6		125.0 ± 22.6		114.2 ± 23.6[y]	
1 (c1)			131.5 ± 24.2	+5.2 % (c0)	137.5 ± 20.0[££]	+10.0 % (c0)	160.1 ± 25.9[££]	+40.2 % (c0)
2 (c2)			134.0 ± 28.9	+1.9 % (c1)	134.4 ± 17.7	−2.3 % (c1)	150.1 ± 27.3	−6.2 % (c1)
3 (c3)			136.0 ± 31.7	+3.4 % (c1)	135.8 ± 17.4	−1.2 % (c1)	157.0 ± 27.8	−1.9 % (c1)
4 (c4)			129.3 ± 20.3	-1.7 % (c1)	137.2 ± 20.3	−0.2 % (c1)	158.5 ± 33.9	−1.0 % (c1)
5 (c5)			128.2 ± 19.2	-2.5 % (c1)	134.5 ± 19.1	−2.2 % (c1)	146.2 ± 28.0[##]	−8.7 % (c1)
10 (c10)			129.0 ± 19.0	-1.9 % (c1)	136.1 ± 21.8	−1.0 % (c1)	145.5 ± 23.1[##]	−9.1 % (c1)
20 (c20)			138.4 ± 33.3	+5.2 % (c1)	134.7 ± 20.7	−2.0 % (c1)	138.5 ± 30.0[##]	−13.5 % (c1)
50 (c50)			137.0 ± 26.6	+4.2 % (c1)	130.5 ± 19.4[##]	−5.1 % (c1)	116.5 ± 40.7[##]	−27.2 % (c1)
100 (c100)			135.3 ± 38.3	+2.9 % (c1)	132.9 ± 17.2	−3.3 % (c1)	135.8 ± 31.7[##]	−15.2 % (c1)
After 3 months	162.4 ± 21.7[££]	+29.9 % (c0)						

[x] Due to technical problems, one sample was excluded from −80 °C salting out method measurement. For this reason, the initial mean concentration in these samples and the standard deviation differ from other salting out protocols.
[y] Due to technical problems in three samples, there was not enough DNA for MagNA liquid nitrogen aliquots, and so additional extraction from frozen blood cells was done. For this reason, the initial mean concentration in these samples and the standard deviation differ from other MagNA protocols.
The data were analysed using Wilcoxon matched-pairs signed-ranks test.
[££] $P < 0.01$, relative to c0 concentration, [*] $P < 0.05$, relative to c1 concentration, [##] $P < 0.01$, relative to c1 concentration

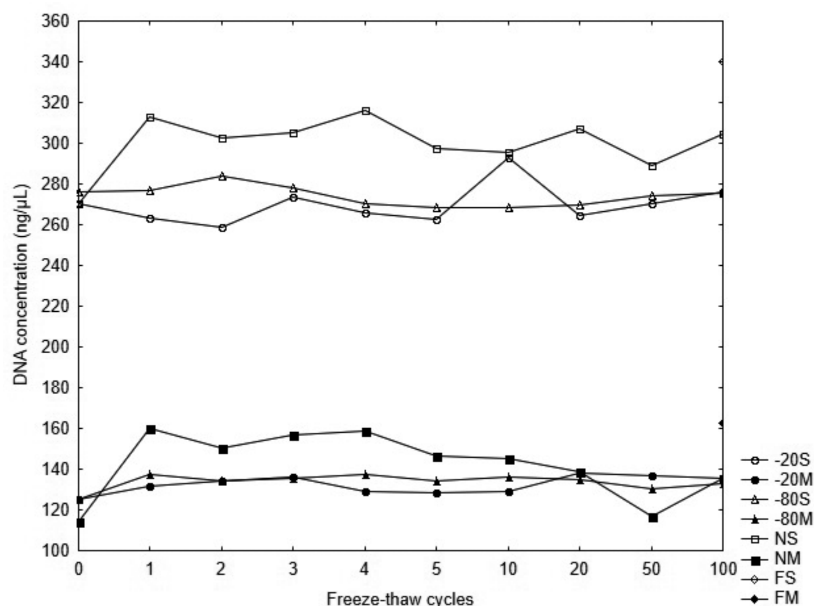

Fig. 1. Concentration changes of DNA isolated by the salting out method and MagNA during freeze/thaw cycles. Concentrations are expressed as mean. **S** = salting out method, **M** = MagNA system**,** **N** = liquid nitrogen, **−20** = −20 °C, **−80** = −80 °C, **F** = refrigerator (2–8 °C). Due to technical problems in three samples, there was not enough DNA for MagNA liquid nitrogen aliquots, and so additional extraction from frozen blood cells was done. For this reason, the initial mean concentration in these samples and the standard deviation differ from other MagNA protocols. Due to technical problems, one sample was excluded from −80 °C salting out method measurement. For this reason, the initial mean concentration in these samples and the standard deviation differ from other salting out protocols.

There were some technical problems during the study: usage of water instead of TE buffer as blank during the quantification and interchanged aliquots. Data from these measurements were excluded from statistical analyses (a total of 13 measurements out of 580 measurements, approx. 2 %). Due to technical problems in two samples, there was not enough DNA for MagNA liquid nitrogen aliquots, and so additional extraction from frozen blood cells was done. Nevertheless, these aliquots were included in the study and following statistical analyses.

Discussion

The biobanks are generally recommended to store DNA samples at either −20 °C or better < −70 °C (Yates, 1989). Both temperatures maintain the DNA integrity (Madisen et al., 1987; Smith and Morin, 2005). In this study, we tested the effect of repeated freezing and thawing on the genomic DNA quality, integrity and concentration to optimize storage procedures used in our biobank.

Although the obtained data showed statistically significant changes in DNA concentrations in some phases of −20 °C and −80 °C freeze/thaw cycling, all deviations except for one (relative to one freeze/thaw cycle concentration) did not exceed ± 5 %, which we consider as acceptable. An outlier value (11 %) was probably the result of a technical problem or careless mixing. Concentration deviations in liquid nitrogen aliquots fluctuated more significantly, however; this could have been

Table 2. Concentration changes per cycle obtained from two-way repeated-measures ANOVA model and MIXED model

Storage	Method	Change per cycle (ng/µl)	P value	95% Confidence Interval
−20 °C	MagNA	0.049	0.587	−0.129–0.227
−20 °C	Salting out	0.078	0.520	−0.162–0.319
−80 °C	MagNA	−0.044	0.483	−0.169–0.080
−80 °C	Salting out	0.088	0.977	−0.174–0.179
Liquid nitrogen	**MagNA**	**−0.274**	**0.009**	**−0.479– −0.069**
Liquid nitrogen	Salting out	−0.072	0.540	−0.304–0.160

Bold = statistical significance

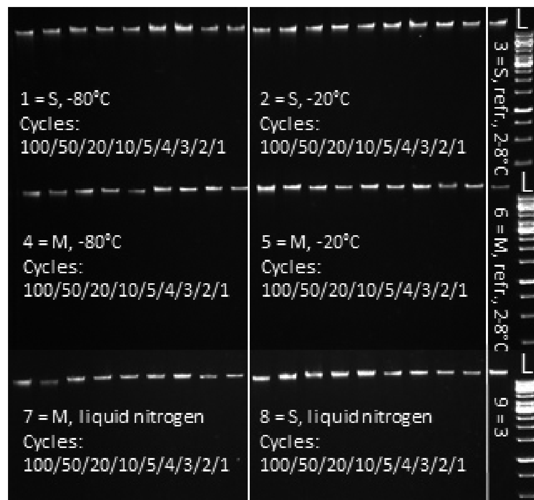

Fig. 2. Electrophoretic separation of isolated DNA. Separation was carried out in 0.7% agarose gel from one volunteer. Bands show freeze/thaw cycles 100/50/20/10/5/4/3/2/1 (from left to right). **1** = salting out method (= S), −80 °C, **2** = salting out method (= S), −20 °C, **3** = salting out method (= S), refrigerator (= refr.) 2–8 °C, **4** = MagNA system (= M), −80 °C, **5** = MagNA system (= M), −20 °C, **6** = MagNA system (= M), refrigerator (= refr.) 2–8 °C, **7** = MagNA system, liquid nitrogen, **8** = salting out method, liquid nitrogen, **9** = 3, **L** = ladder, GeneRuler 1kb DNA Ladder (Fermentas).

caused by insufficient mixing and handling of thawed samples. The concentration changes in MagNA samples were higher compared to the salting out samples (statistically significant). The repeated freeze/thaw cycles influenced neither integrity, nor purity of genomic DNA. Based on the obtained data, storage of gDNA at both −20 °C and −80 °C in high-volume aliquots seems to be convenient and results in storage cost saving. We do not consider liquid nitrogen to be a suitable storage means because the way of mixing the thawed samples can significantly influence their measured concentration.

It is worthwhile to mention some limitations of our study. We optimized our laboratory processes, and so the presented results reflect our routine laboratory conditions and samples (source of DNA, extraction methods, DNA concentration, dilution buffer, etc.). Therefore, it may be assumed that changes in the study design (e.g., use of low DNA concentration) could lead to different results.

During concentration measurements, we clearly noticed the need for careful mixing of thawed samples (according to how the DNA solution behaved during mixing using a pipette tip), suggesting that low temperatures lead to formation of DNA clumps, especially during liquid nitrogen storage.

Previous studies have tested the effect of repeated freeze/thaw cycles on DNA quality. However, their observations were contradictory. On the one hand, Ross et al. (1990) demonstrated stability of DNA in repeated frozen/thawed samples (a total of 40 cycles and five donors). They compared samples stored at 4 °C with samples repeatedly frozen and thawed. The DNA quality was subsequently determined by both OD 260/280 and fingerprinting. These experiments included samples isolated using manual phenol/chloroform extraction with ethanol precipitation (Ross et al., 1990). On the other hand, Shao et al. (2012) evaluated samples extracted using a QIAmp Blood Maxi Kit, Gentra Puregene Blood Kit, and manual phenol/chloroform method with ethanol precipitation. The results of their experiments showed progressive degradation of DNA as the number of freeze/thaw cycles increased (a total of 18 cycles and 10 donors). The decreasing quality of genomic DNA was demonstrated using pulsed field gel electrophoresis. They tested two concentrations of DNA, 10 μg/ml (= 10 ng/μl) and 100 μg/ml (= 100 ng/μl) for degradation. The study also observed that DNA with higher concentration was better protected from degradation. For comparison, our average initial concentrations were higher, 125.0 ng/μl and 270.6 ng/μl, respectively.

In conclusion, the repeating freeze/thaw cycles (through −20 °C and −80 °C, respectively) do not significantly influence the integrity, concentration, or purity of genomic DNA, which allows storage of samples in high-volume pools without multiple aliquoting. We consider storage in a freezer to be the most suitable way of long-term DNA preservation. Liquid nitrogen storage leads to formation of DNA clumps, and the concentration of samples stored in a refrigerator increases very significantly.

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Conflict of interest

The authors declare that they have no conflict of interest.

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