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Importance of quality control in sample preparation workflow of forensic degraded samples for massively parallel sequencing (MPS)

Viktorija Belakaposka Srpanova[1*], Sasho Risteski[1], Natasha Bitoljanu[1], Aleksandar Stankov[1], Zlatko Jakovski[1]

[1]Institute of Forensic Medicine, Criminalistics and Medical Deontology, Faculty of Medicine, Ss. Cyril and Methodius University in Skopje

* Corresponding author: belakaposka@yahoo.com

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Abstract

Molecular genetic analyses occupy a significant part of laboratory tests in forensic practice. Pre-prepared commercial kits used for DNA extraction are an excellent choice for samples provided by living people, but in forensic practice, most of the samples that undergo molecular processing are often degraded and may pose a challenge in the laboratory. A special challenge is to provide quality genetic material from post-mortem samples that would be suitable for further analysis with massively parallel sequencing. The aim of this paper is to evaluate the applicability of two silica-based DNA extraction methods for post-mortem blood samples with varying storage time and degrees of degradation, and also, to establish proper quality control in sample preparation workflow for Massively Parallel Sequencing using Ion Torrent GeneStudio™ S5 platform. The study was performed on 48 blood samples with different storage time. Silica-based DNA extraction protocols was applied and quantification was made using Qubit 3.0 fluorimeter, followed by Real-Time PCR quantification. The results indicate high variability between the obtained DNA quantities from post-mortem blood samples with different storage time. Correlation was determined between storage time and sample quantity and quality. The Qiagen Mini Kit & Micro Kit are applicable for DNA extraction from blood samples with various storage periods and degradation levels, which can be used for further NGS analyses after careful quality control and optimization of library preparation conditions.

Introduction

A molecular autopsy is becoming an essential part of forensic practice in the past years [Castiglione]. A special challenge in forensic medical research are pathologies that are not encountered very often, thus limit the availability of research samples. In order to form an eligible sample group, researchers rely on aged and/or degraded samples, obtained and stored for different use and not always adequate for new emerging techniques. Nowadays, pathology of high interest for a forensic medical researcher is sudden cardiac death (SCD), especially in case of a negative autopsy (the absence of any structural change and negative histological and toxicological tests). Since SCD and negative autopsy pose a strong indicator of the presence of an inherited pathology of the heart, genetic testing is suggested [Torkamani]

Due to long storage time and degradation, the quality of biological samples is reduced [Madisen]. DNA analysis methods typically used in forensic medicine are based on short DNA fragments that are easily amplified even from biological samples with a high degree of degradation [Bukyya]. Unlike typical forensic analysis, massively parallel sequencing requires biological samples of higher quantity and quality.

Massively parallel sequencing (MPS) is a high-throughput method for DNA sequencing that enables the simultaneous analysis of millions of sequences

[Nagy]. The quality of the starting sample is critical for the success of MPS, as poor-quality samples can lead to inaccurate and unreliable results. MPS platforms typically require high molecular weight DNA with minimal degradation and chemical modifications, and high purity and concentration. If the starting sample is of low quality, the sequencing library may contain biased or low-quality sequences, which can lead to errors, false positives, and reduced accuracy [Ug MAN0013432]. The quality of the starting sample can be assessed using various methods, such as gel electrophoresis, spectrophotometry, or fluorometry. Gel electrophoresis can be used to visualize the DNA fragmentation and assess the integrity, while spectrophotometry and fluorometry can be used to determine the concentration of the DNA, with the spectrophotometry additionally being able to detect chemical impurities [Nielsen]. Various pre-sequencing sample preparation protocols can be used depending on the type and quality of the starting sample, since this can affect the success of the consecutive steps such as DNA fragmentation, size selection, and adapter ligation. Overall, the quality of the starting sample is critical for the success of NGS, and careful sample preparation and quality control measures are essential to ensure accurate and reliable results.

The aim of the study is to determine the correlation between the storage time of post-mortem blood samples and extracted DNA quantity and quality, and to evaluate DNA sample eligibility for successive MPS analyses. Also, the aim is to establish proper quality control in sample preparation workflow for Massively Parallel Sequencing using Ion Torrent GeneStudio™ S5 platform.

Materials and methods

We analysed 48 post-mortem blood samples (peripheral whole blood without anticoagulant) with different storage time, short term (1-3 months) at 4 °C, followed by long term storage (3-122 months) at -20 °C. The analysed samples were taken from forensic-medical autopsies performed in a 10-year period (2013-2022) at the Institute for Forensic Medicine, Criminology and Medical Deontology at the Faculty of Medicine, St. Cyril and Methodius University in Skopje. All samples were obtained according to the standard protocols for forensic autopsy, biological sample processing and storage prescribed by the European Council of Forensic Medicine and R99 recommendations [Harmonization].

DNA extraction from peripheral whole blood

Blood used for DNA extraction was taken directly from a blood vessel of the deceased during autopsy and collected in sterile blood collection tubes without anticoagulant.

Two genomic DNA extraction protocols were applied. Eleven samples were extracted by using QIAamp DNA Micro Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions, except for the starting volume of blood, which was increased from 100 µl to 200 µl, along with proportionally increasing the volume of lysis buffers. DNA was eluted in 100 µl of TE buffer. The rest of the samples (37) were extracted by using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions, except for the elution volume, which was reduced from 200 µl to 100 µl.

DNA quantification

After DNA extraction, quantitation was initially done by using Qubit™ dsDNA HS Assay Kit on the Qubit 3.0 fluorimeter. Ten microliters of the DNA eluate were used for quantification.

To evaluate the amount of amplifiable DNA extracted from different post-mortem blood samples by Real-Time PCR, we used the Quantifiler™ Trio DNA Quantification Kit (Thermofisher Scientific) on the 7500 Real-Time PCR System, according to manufacturer's instructions. The kit also gives indication of the degradation of the DNA, by comparing the amplification success of one small and one large target. The quantity of the DNA samples was estimated by using the quantity of the small autosomal target.

Results and Discussion

The results of the performed quantity measurements of DNA are shown in Table 1.

Table 1. DNA quantification results.

Sample No.	Storage time (months)	Extraction kit used	Qubit quantification (ng/µl)	qPCR quantification of Small Autosomal Target (ng/µl)	qPCR quantification of Large Autosomal Target (ng/µl)	Degradation Index
1	1	Mini	10,4	46,22	31,509	1,467
2	1	Mini	10,2	51,116	38,17	1,339
3	1	Mini	10,4	98,573	62,892	1,567
4	1	Micro	9,46	17,978	16,22	1,108
5	6	Mini	124	160,902	46,821	3,437
6	7	Mini	5,68	8,084	9,885	0,818
7	10	Mini	10,6	145,12	137,526	1,055
8	13	Micro	9,14	10,362	9,682	1,07
9	18	Micro	9,6	13,41	16,742	0,801
10	19	Micro	11	41,744	32,87	1,27
11	24	Micro	10,8	29,794	31,358	0,95
12	24	Micro	10	23,911	24,842	0,963
13	26	Mini	0,116	0,049	0,003	16,333
14	29	Mini	4,82	5,041	7,552	0,668
15	30	Micro	6,18	7,867	10,811	0,728
16	30	Mini	4,28	3,318	2,284	1,453
17	49	Mini	4,86	0,195	0,14	1,393
18	49	Mini	8,42	3,238	3,653	0,886
19	50	Mini	0,065	0,018	0,01	1,8
20	60	Mini	1,63	1,483	0,788	1,882
21	60	Mini	5,32	4,879	6,439	0,758
22	60	Mini	2,38	2,513	1,61	1,561
23	60	Mini	0,436	0,276	0,059	4,678
24	60	Mini	4,42	7,405	6,663	1,111
25	60	Mini	5,14	9,103	10,216	0,891
26	72	Mini	4,44	5,117	2,526	2,026
27	72	Mini	2,1	2,367	2,002	1,182
28	72	Mini	2,18	1,747	1,612	1,084
29	72	Mini	0,72	0,502	0,109	4,606
30	72	Mini	1,73	0,223	0,051	4,373
31	84	Mini	0,474	0,128	0,117	1,094
32	84	Mini	0,37	0,034	0,002	17
33	84	Mini	1,61	1,192	0,42	2,838
34	96	Mini	3,28	2,74	2,056	1,333
35	96	Mini	4,3	1,423	0,054	26,352
36	96	Mini	6,72	13,915	7,834	1,776
37	96	Mini	2,02	2,078	1,25	1,662
38	96	Mini	1	0,409	0,161	2,54
39	96	Mini	0,894	0,866	0,288	3,007
40	106	Micro	0,594	0,483	0,346	1,396
41	118	Mini	0,804	0,544	0,211	2,578

42	120	Mini	3,42	2,7	2,535	1,065
43	120	Mini	2,02	1,698	1,796	0,945
44	120	Mini	2,16	1,27	0,851	1,492
45	120	Mini	1,63	1,177	0,425	2,769
46	121	Micro	3,12	2,747	2,648	1,037
47	122	Micro	1,27	1,352	1,449	0,933

DNA was extracted using two extraction kits (QIAamp DNA Micro Kit & QIAamp DNA Mini Kit) and quantity was measured by fluorometric and Real-Time PCR approach. The results indicate high variability between the obtained DNA quantities from post-mortem blood samples with different storage time. This means that the storage time is probably not the only factor affecting the DNA quantity, and that other circumstances can influence the overall yield. Although some samples had low DNA yield, it was shown that silica-based DNA extraction method, such as QIAamp DNA Micro/Mini Kit, is adequate even for aged samples. Previous studies have also shown the applicability of silica-based DNA extraction methods for samples that have been stored for several years [Tagliaferro].

The maximum DNA concentration in our research was 160,902 ng/µl, obtained from sample kept for 6 months, extracted with the Mini Kit, as for the lowest concentration with the same kit was 0,018 ng/µl, from sample kept for 50 months. The highest concentration obtained with Micro Kit is 41,744 ng/µl from sample kept for 19 months, and the lowest with the Micro kit was 0,483 ng/µl from sample kept for 106 months.

Many modern applications in molecular biology require pure and intact genomic DNA as a first choice. Therefore, the efficiency of a DNA extraction method will be affected by its robustness and ability to yield optimal amount of clean and intact genomic DNA. The total count of WBC in a normal blood in adult human is ranged from 4.5-10 × 103/µL, thus the total amount of DNA per µL of blood has been calculated to be within the range of 29.48-65.5 ng approximately [Guha]. In our research, only 14.5 % of samples fit in this range, all of them being stored for less than 24 months. The specimens with longer storage fell far behind the quantity and quality of fresh blood samples.

When comparing the DNA concentration of the blood samples kept for 1 year, the highest noted concentration was 160,902 ng/µl (as measured by qPCR) and 124 ng/µl (as measured by Qubit). The highest noted concentration from the samples kept for 10 years is 2,747 ng/µl (as measured by qPCR) and 3,12 ng/µl (as measured by Qubit).

Statistical analyses were performed to check for correlation between storage time and quantity of extracted DNA (Figures 1-3). By applying the Pearson correlation coefficient, it was determined that there is a significant negative relationship between the duration of storage of the blood samples and the obtained concentration of DNA measured with the Qubit

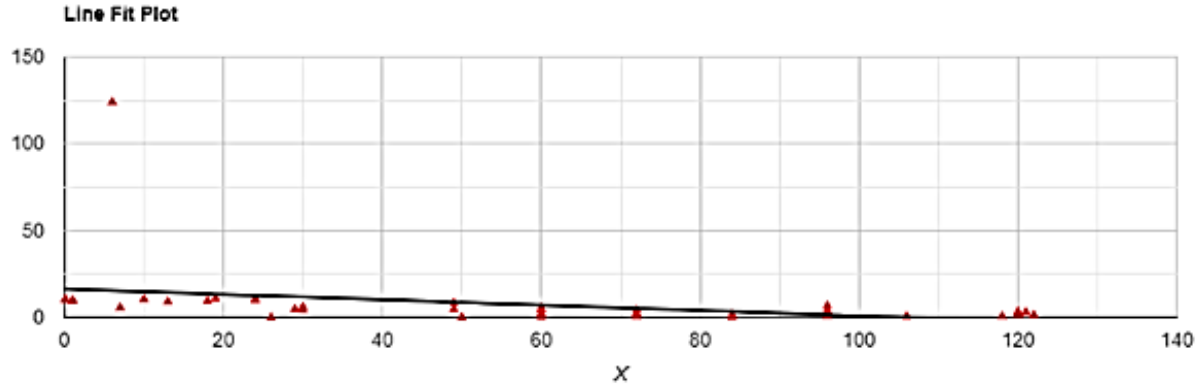

Figure 1. Pearson correlation coefficient Qubit concentration vs. storage time.

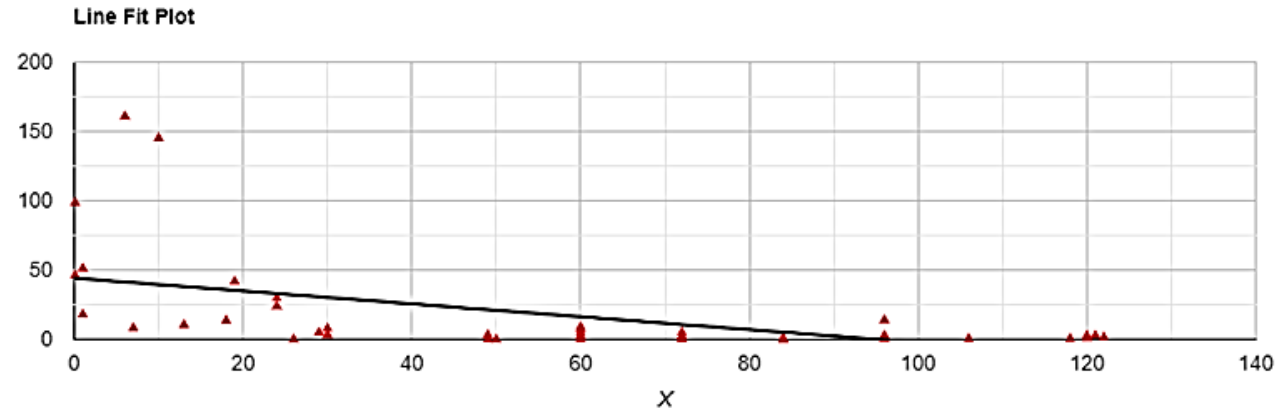

Figure 2. Pearson correlation coefficient between the small autosomal target DNA concentration vs. storage time.

fluorometer, i.e., the longer the storage time of the material, the lower the concentration of isolated DNA in a blood sample. (r2= -0.346, p=0.017).

By applying the Pearson correlation coefficient, it was determined that between the duration of storage of the blood samples and the concentration of the small autosomal target obtained by Real-Time PCR, there is a significantly negative dependence, that is, the longer the time of storage of the sample, the lower the concentration of the small DNA fragment in a blood sample (r2= -0.536, p < 0.001).

By applying the Pearson correlation coefficient, it was determined that between the duration of storage of the material and the concentration of the long fragment obtained by PCR, there is a significantly negative dependence, i.e. the longer the time of storage of the material, the concentration of the long DNA fragment in a blood sample is lower (r2= -0.550, p < 0.001)

By applying the Pearson correlation coefficient, it was determined that the measured concentration between the Qubit and Quantifiler Trio DNA Quantification Kit, there is a positive correlation, which means there is a tendency for high Qubit measured concentration to go with high Quantifiler Trio DNA Quantification Kit (and vice versa). (r2= 0.7063, p < 0.0001)

Comparing the results obtained from different quantification methods, some deviation has been noted. Namely, in some samples, fluorometric measurement overestimated or underestimated the quantity of the extracted DNA. The high variability between the obtained DNA quantities, were incentive to perform additional estimation of the DNA quantity and quality by Real-time PCR, even though most of the

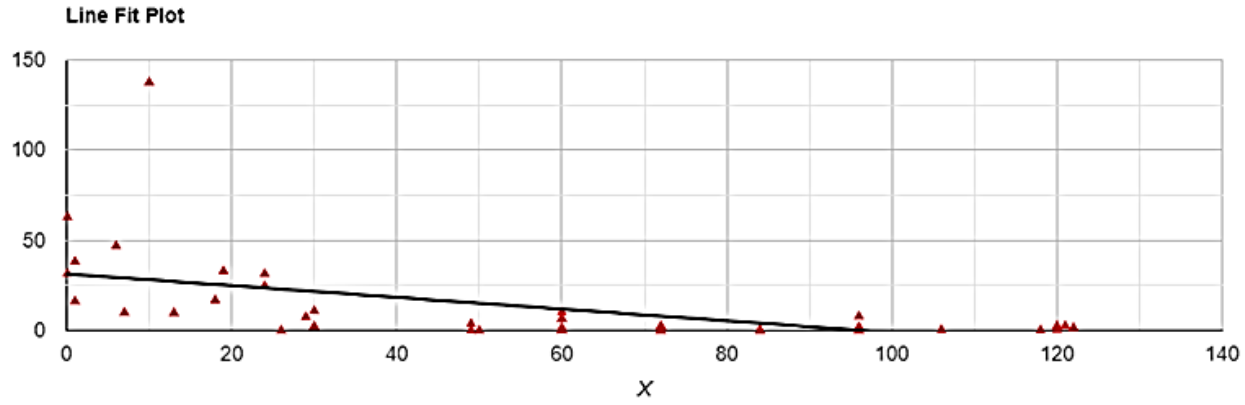

Figure 3. Pearson correlation coefficient between the large autosomal target DNA concentration vs. storage time.

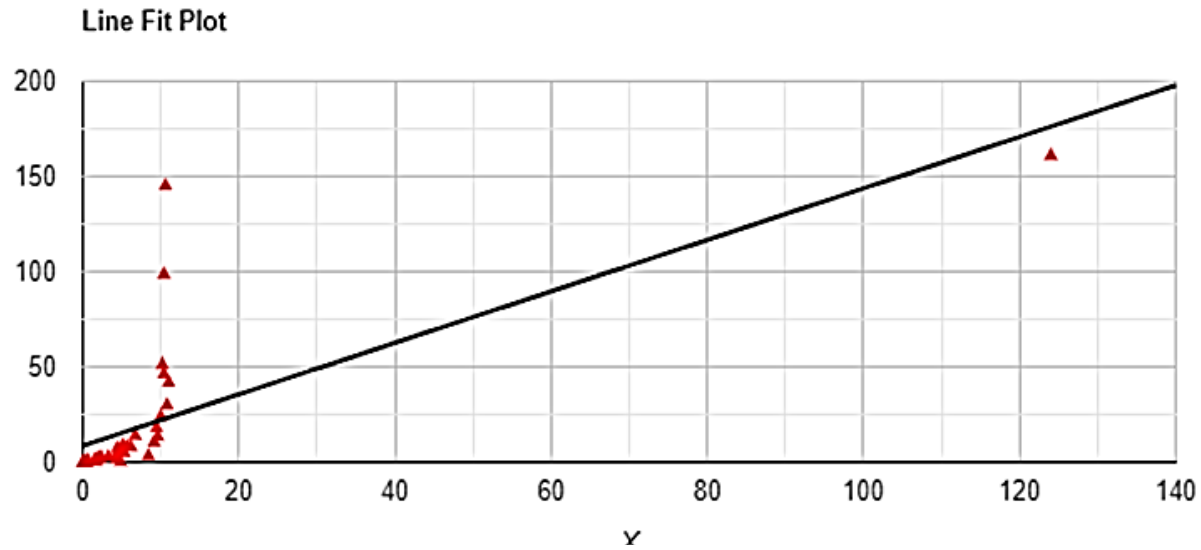

Figure 4. Pearson correlation coefficient Qubit concentration vs. Quantifiler Trio DNA Quantification Kit.

library preparation workflows used, such as Precision ID DL8 Kit (Applied Biosystems™) in Ion Torrent S5 sequencing, do not require precise DNA quantification by Real-Time PCR [Ug MAN0013432].

The Quantifiler Trio DNA Quantification Kit which was used in this study, measures total DNA present and inhibitors in a DNA sample by measuring the quantity of one short DNA fragment, one long DNA fragment and one internal positive control. The kit can be used to establish a degradation index of the DNA (useful in routine forensic DNA typing) [Ug 4485354].

The deviation between the values determined by Qubit can be attributed to different factors, such as the variable degradation levels of the samples and the possible inhibition in the samples.

The Qubit HS dsDNA assay is regarded as a highly specific and sensitive DNA quantification assay and is preferred when quantifying DNA for downstream NGS analysis. However, when dealing with sample sets with different DNA integrity and possible presence of PCR inhibitors, overestimation or underestimation of the DNA concentration can arise. Library preparation workflow requires equal starting DNA input in order to achieve equal amplification of all gene targets, and thus to provide complete gene coverage for all of the samples. Also, when the measured DNA concentration is overestimated / underestimated, the PCR amplification of the overloaded samples will be more efficient than the rest of the samples, thus the final library and the sequencing chip will be oversaturated with amplicons from the overloaded samples, which in turn can result with poor coverage of the rest of the samples. This is particularly difficult in MPS platforms that utilize automated library preparation (such as Thermofischer's Ion S5 Sequencer coupled with Chef Instrument for library preparation, template preparation and chip loading), which do not allow for individual library quantification, but provide an 8-samples library pool which can be then diluted to the optimal concentration [Ug 4485354]. According to our experience, this issue can be avoided only with the use of real-time PCR quantification step before library preparation and diluting samples to equal DNA quantity to ensure equal input in library preparation reactions.

Conclusions

The Qiagen Mini Kit & Micro Kit are applicable for DNA extraction from aged blood samples with various storage periods and degradation levels.

Qubit measurement is recommended for assessment of DNA concentration by library preparation kit's manuals, however our study showed that total DNA measured by Qubit should be taken with caution, and when possible, real-time PCR quantification should be performed in

order to establish the total amplifiable DNA in the sample.

Older samples sometimes tend to have higher degradation levels, thus, the DNA input and cycle numbers in further steps in library preparation workflow should be adjusted carefully to achieve optimal library concentration for subsequent sequencing.

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